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Antibiotic A 40926 complex and its pure factors PA, PB, A, B and BO.

The present invention concerns a new antibiotic substance arbitrarily denominated antibiotic A 40926 complex and its factors antibiotic A 40926 factor A, antibiotic A 40926 factor B, antibiotic A 40926 factor B<sub>0</sub>, antibiotic A 40926 factor PA and antibiotic A 40926 factor PB, a process for producing them by culturing the new strain *Actinomadura* sp. ATCC 39727 or an A 40926 producing variant or mutant thereof and the use of these new antibiotic substances in the treatment of infectious diseases involving microorganisms susceptible to them. The antibiotic substances of the invention are glycopeptidic substances belonging to the vancomycin class or antibiotics which have binding capacity to Acyl-D-Alanyl-D-Alanine.

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ANTIBIOTIC A 40926 COMPLEX  
AND ITS PURE FACTORS PA, PB, A, B AND B<sub>0</sub>

10 The present invention concerns a new antibiotic  
substance arbitrarily denominated antibiotic A 40926  
complex and its factors antibiotic A 40926 factor A,  
antibiotic A 40926 factor B, antibiotic A 40926 factor  
B<sub>0</sub>, antibiotic A 40926 factor PA and antibiotic A 40926  
15 factor PB, a process for producing them by culturing the  
new strain Actinomadura sp. ATCC 39727 or an A 40926  
producing variant or mutant thereof and the use of these  
new antibiotic substances in the treatment of infectious  
diseases involving microorganisms susceptible to them.

20 The antibiotic substances of the invention are  
glycopeptidic substances belonging to the vancomycin  
class of antibiotics which have binding capacity to  
Acyl-D-Alanyl-D-Alanine.

25 Antibiotic A 40926 complex and its factors antibiotic  
A 40926 factor A, factor B, factor B<sub>0</sub>, factor PA and  
factor PB, may form salts according to known per se  
techniques.

In the present description and claims the expression  
30 "antibiotic A 40926" as such represents a compound  
selected from antibiotic A 40926 complex, antibiotic  
A 40926 factor A, antibiotic A 40926 factor B,  
antibiotic A 40926 factor B<sub>0</sub>, antibiotic A 40926 factor  
PA, antibiotic A 40926 factor PB a salt thereof or any  
35 mixture thereof. The expression "antibiotic A 40926"

complex", "antibiotic A 40926 factor PA", "antibiotic A 40926 factor PB", "antibiotic A 40926 factor A", "antibiotic A 40926 factor B", antibiotic A 40926 factor B<sub>0</sub>, when dealing with the biological properties, will  
5 encompass also the corresponding pharmaceutically acceptable salts.

Antibiotic A 40926 is produced by cultivating an Actinomadura strain isolated from a soil sample and  
10 which has been deposited on June 8, 1984 at the internationally recognized collection American Type Culture Collection (ATCC) - 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty. The strain has been accorded the  
15 accession number ATCC 39727.

The producing strain was originally thought to belong to the genus Streptomyces and was initially denominated Streptomyces nov. sp. A 40926 and as such deposited with  
20 ATCC where it received the accession number ATCC 39727. Further studies, especially on cell-wall composition brought us to conclude that the new strain belongs to the genus Actinomadura. Accordingly, the strain originally deposited as Streptomyces sp. ATCC 39727 has  
25 been renamed Actinomadura sp. ATCC 39727.

The production of antibiotic A 40926 complex, antibiotic A 40926 factor A, factor B, factor B<sub>0</sub>, factor PA, or factor PB, is achieved by cultivating an Actinomadura  
30 sp. capable of producing it, i.e. Actinomadura sp. ATCC 39727 or an antibiotic A 40926-producing variant or mutant thereof, under aerobic conditions in an aqueous nutrient medium containing assimilable sources of carbon, nitrogen, and inorganic salts. Many of the  
35 nutrient media usually employed in the fermentation art,

can be used, however certain media are preferred.

Preferred carbon sources are glucose, mannose, galactose, starch, corn meal and the like. Preferred nitrogen sources are ammonia, nitrates, soybean meal,

5 peptone, meat extract, yeast extract, tryptone, aminoacids, and the like. Among the inorganic salts which can be incorporated in the culture media there are the customary soluble salts capable of yielding sodium, potassium, iron, zinc, cobalt, magnesium, calcium,  
10 ammonium, chloride, carbonate sulfate, phosphate, nitrate and the like ions.

Ordinarily, the antibiotic-producing strain is pre-cultured in a shake flask, then the culture is used to inoculate jar fermentors for production of

15 substantial quantities of the antibiotic substances. The medium used for the pre-culture can be the same as that employed for larger fermentations, but other media can also be employed. The antibiotic A 40926

20 producing-strain can be grown at temperatures between 20 and 40°C, preferably between 24 and 35°C.

During fermentation, the antibiotic production can be monitored by testing broth or mycelial extract samples for antibiotic activity for instance by bioassays or TLC or HPLC procedures.

25 Sensitive organisms to antibiotics A 40926 such as Bacillus subtilis and S. aureus can be used as test organisms. The bioassay is conveniently performed by the agar diffusion method on agar plates. Maximum production of antibiotic activity generally occurs between the  
30 second and the fifth day of fermentation.

Antibiotic A 40926 is produced by cultivating the strain Actinomadura sp. ATCC 39727, or an antibiotic A 40926 producing mutant or variant thereof, and is mainly found in the culture broths.

The characteristics of Actinomadura sp. A 40926  
ATCC 39727 are given in the following paragraphs:

Macroscopic and microscopic examination

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The vegetative mycelium is composed of flexuous and branched hyphae (about 0.8  $\mu$ m of diameter) which on some media, identified by an asterisk in Table I, slightly tends to fragment into rod-like elements after several days of growth, while on glucose-asparagine medium it fragments into coccoid elements.

10

Characteristic of this strain is the Burgundy color of the vegetative mycelium on some media.

15

The aerial mycelium is present only in few media; in particular, among those listed in Table I, it is present only in oatmeal agar and soil agar. On these media the aerial mycelium is white-grey and forms sporophores arranged in hooks and short spirals of about 10 to 20 spores.

20

The spores are cylindrical and have an average size of 0.8 x 1.2  $\mu$ m.

Determination of growth characteristics

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For the examination of the cultural characteristics, Actinomadura sp. ATCC 39727 was cultivated on various standard media suggested by Shirling and Gottlieb (Shirling E.B. and Gottlieb D., 1966 - Method for

30

characterization of Streptomyces species - Int. J. Syst. Bacteriol, 16, 313-340) with the addition of several media recommended by Waksman (Waksman, S.A.: 1961 - The Actinomycetes - The Williams and Wilkins Co. Baltimore; Vol. 2, 328-334).

Color determination was made whenever necessary by the method of Maerz and Paul (Maerz A. and M. Rea Paul, 1950 - A Dictionary of Color - 2nd Edition McGraw-Hill Book Company Inc. New York).

- 5 The ability of the organism to utilize different carbon sources was investigated by the method described by Shirling and Gottlieb.

The cultural and physiological characteristics and the carbon sources utilization are reported in Tables I, II,

- 10 III.

The readings in Table I have been taken after two weeks incubation at 28°C.

TABLE I  
CULTURAL CHARACTERISTICS OF STRAIN  
Actinomadura sp. ATCC 39727

| Culture media  | Characteristics  |
|--|--|
| Medium No. 2<br>(yeast extract<br>- malt agar)         | Abundant growth, with crusty<br>surface, 8/L/8, traces of<br>amber-pink soluble pigment  |
| Medium No. 3<br>(oatmeal agar)                         | Abundant growth, with smooth<br>surface, violet, 55/E/4,<br>aerial mycelium very scant<br>grey, soluble pigment violet<br>55/H/4 |
| Medium No. 4<br>(inorganic salts-<br>starch agar)      | Moderate growth, with smooth<br>and thin surface, cream<br>10/D/2  |
| Medium No. 5<br>(glycerol-asparagine<br>agar)          | Moderate growth, with smooth<br>and thin surface, apricot<br>10/B/2  |
| Medium No. 6 *<br>(peptone-yeast extract<br>iron agar) | Moderate growth, with<br>slightly crusty surface,<br>amber 12/D/9  |
| Medium No. 7<br>(tyrosine agar)                        | Abundant growth, with smooth<br>and thin surface,<br>amber-brown 13/K/12   |
| Oatmeal agar *   | Abundant growth, with smooth<br>surface, Burgundy 8/L/7,<br>aerial mycelium, moderate<br>light yellow-grey 44/B/2                |
| Hickey and<br>Tresner's agar *                         | Abundant growth, with<br>wrinkled surface,<br>amber-brown 13/K/12  |

TABLE I (Continued)

| Culture media             | Characteristics  |
|---------------------------|--|
| Czapeck glucose agar      | Moderate growth, with smooth surface, light-yellow 9/I/3                                     |
| Glucose asparagine agar * | Scant growth, with creamy surface, straw-yellow 9/E/1  |
| Nutrient agar             | Abundant growth, with wrinkled surface, light-orange 11/C/7                                  |
| Potato agar *             | Abundant growth, with wrinkled surface, Burgundy 8/L/9                                       |
| Bennett's agar *          | Abundant growth, with crusty surface, Burgundy 8/L/8, soluble pigment deep amber rose 5/J/10 |
| Calcium malate agar       | Moderate growth, with smooth surface, apricot 10/B/3   |
| Skim milk agar            | Abundant growth, with slightly wrinkled surface, orange 9/B/9                                |
| Czapeck sucrose agar      | Abundant growth, with smooth surface, apricot 10/B/6   |
| Egg albumin agar *        | Abundant growth, with smooth surface, rose 52/B/3, traces of a soluble pigment, rose 52/B/3  |



TABLE I (Continued)

| Culture media            | Characteristics   |
|--------------------------|---|
| Sabouraud agar           | No growth   |
| Soil agar                | Scant growth, colorless,<br>white-grey aerial mycelium                    |
| Dextrose triptone agar * | Moderate growth, with smooth<br>and thin surface,<br>light-yellow 10/G/2  |
| Potato plug              | Abundant growth,<br>orange-brown, traces of<br>white-grey aerial mycelium |

Physiological characteristics

TABLE II  
Physiological characteristics

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| Tests                      | Results   |
|----------------------------|---|
| Starch hydrolysis          | negative  |
| H <sub>2</sub> S formation | negative on Medium No. 6<br>positive with lead acetate strips |
| Tyrosine reaction          | positive  |
| Casein hydrolysis          | positive  |
| Calcium malate hydrolysis  | negative  |
| Gelatin liquefaction       | positive  |
| Litmus milk                | coagulation negative<br>peptonization positive                |
| Cellulose decomposition    | negative  |
| Nitrate reduction          | positive  |

Utilization of carbon sources

TABLE III  
Carbon Utilization

5

|    | Carbon Source | Growth |
|----|---------------|--------|
| 10 |               |        |
|    | Arabinose     | +      |
|    | Xylose        | +      |
|    | Mannose       | +      |
| 15 | Fructose      | +      |
|    | Raffinose     | +      |
|    | Rhamnose      | +      |
|    | Glucose       | +      |
|    | Lactose       | +      |
| 20 | Galactose     | +      |
|    | Inositol      | -      |
|    | Sucrose       | +      |
|    | Cellulose     | -      |
|    | Salicin       | +      |
| 25 | Mannitol      | +      |
|    | Ribose        | -      |

+ = growth

30 - = no growth

Chemotaxonomical characteristicsCell wall analysis:

- 5 The analysis of aminoacids present in the cell wall was carried out by the methods described in the work of Becker et al., "Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole cell hydrolysates", *Appl. Microbiol.* 12, 421-423 (1964).
- 10 The analysis of the whole cell hydrolyzed revealed the presence of meso-diaminopimelic acid.
- The analysis of pure cell wall, obtained with the method of Kawamoto et al. (I. Kawamoto, T. Oka, and T. Nara, "Cell-wall composition of Micromonospora
- 15 olivoasterospora, Micromonospora sagamiensis, and related organism", *J. of Bacteriology* 146, 527-534, 1981) showed absence of glycine.

Sugar analysis:

20

- The analysis of sugar content was carried out by the method of M.P. Lechevalier, "Identification of aerobic actinomycetes of clinical importance", *J. Lab. Clin. Med.* 71, 934-944 (1968) using thin layer chromatography
- 25 cellulose sheets as described by J.L. Staneck and G.D. Roberts, "Simplified approach to identification of aerobic actinomicetes by thin-layer chromatography", 28, 226-231 (1974) with the following solvent system:
- Ethylacetate-Pyridine-Water (100 : 35 : 25 by volume).

30

- The obtained results showed the presence of mainly glucose and ribose while lower quantities of galactose, mannose and madurose (3-O-methyl-D-galactose) were also detected.

Mycolic acids:

- An assay for detecting the presence of mycolic acids was carried out by the following method of Minnikin et al. (D.E. Minnikin, L. Alshamaony and M. Goodfellow, "Differentiation of Mycobacterium, Nocardia and related taxa by thin layer chromatography analysis of whole organism methanolysates", Journal of General Microbiology 88, 200-204, 1975).
- 10 The results of the assay were negative: mycolic acids were not found.

Identity of the strain:

- 15 The strain is assigned to the Actinomycetes genus Actinomadura because of the presence of meso-diaminopimelic acid and madurose, the lack of glycine in the peptidoglycan, the lack of mycolic acids and the formation of aerial mycelium with moderately
- 20 long spore chains.

- As with other microorganisms, the characteristics of the
- 25 A 40926 producing strain are subject to variation. For example, artificial variants and mutants of the strain can be obtained by treatment with various known mutagens, such as U.V. rays, X-rays, high frequency waves, radioactive rays, and chemicals such as nitrous acid, N-methyl-N'-nitro-N-nitrosoguanidine, and many
- 30 others. All natural and artificial variants and mutants which belong to the species of the genus Actinomadura and produce A 40926 antibiotics, are deemed equivalent to strain Actinomadura sp. ATCC 39727 and are
- 35 contemplated to be within the scope of this invention.

The recovery of the antibiotic substances of the invention from the fermentation broths of the producing microorganism is conducted according to known per se techniques which include extraction with solvents, precipitation by adding non-solvents or by changing the pH of the solution, partition chromatography, reverse-phase partition chromatography, ion-exchange chromatography, affinity chromatography and the like.

- 5  
10 A preferred procedure includes an affinity chromatography on immobilized D-Alanyl-D-Alanine followed by reverse-phase column chromatography.

- 15 Immobilized D-Alanyl-D-Alanine matrices suitable for the present recovery process are disclosed in European Patent Application No. 83112555. The preferred matrix in the present process is D-Alanyl-D-alanine coupled with a controlled pore cross-linked polydextrane.

- 20 The fermentation broth can be subjected to the affinity chromatography directly after filtration or after a preliminary purification procedure. This latter procedure includes making the whole fermentation mass basic, preferably between pH 8.5 and 10.5, in order to  
25 solubilize the antibiotic substance adsorbed on the mycelium and then filtering. The clear filtrate is brought to pH 2.5-4.5 and filtered again in the presence of a filter aid. This filtrate is discarded while the recovered filtration cake is suspended in water, made basic, preferably at a pH between 8 and 9, and filtered. The filtration cake is re-subjected to the same treatment procedure while the filtrates, which contain antibiotic substances, are pooled.

These filtrates or the filtered fermentation broths are then subjected to an affinity chromatography on immobilized D-Alanyl-D-Alanine, either in column or batchwise.

- 5 The binding of the antibiotic substance to the affinity matrix is preferably made at a pH of about 7.0-8.0 and its elution is performed at more basic pH values (preferably between 9.0 and 10.5) by means of an aqueous base. This aqueous base may be ammonia, a volatile  
10 amine, an alkali or alkali metal hydroxide or a basic buffered solution optionally in the presence of a polar organic solvent such as a polar water-miscible solvent as defined below.

- After removing the impurities by rinsing the column with  
15 aqueous buffer pH 4-8, optionally containing salts, urea and/or water miscible solvents, the antibiotic A 40926 is eluted with the above eluting mixture. The crude antibiotic substance is then recovered preferably by removing water from the pooled antibiotic-containing  
20 fractions by azeotropical distillation with an organic solvent capable of forming minimum azeotropic mixtures with water, followed by addition of a non-solvent to precipitate the desired product.

- Representative examples of organic solvents capable of  
25 forming minimum azeotropic mixtures with water are n-butanol, benzene, toluene, butyl ether, carbon tetrachloride, chloroform, cyclohexane, 2,5-dimethylfuran, hexane and m-xylene; the preferred solvent being n-butanol.

- 30 Examples of non-solvents are: petroleum ether, lower alkyl ethers, such as ethyl ether, propyl ether and butyl ether, and lower alkyl ketones such as acetone. Alternatively, the pooled antibiotic-containing fractions are concentrated to a small volume, preferably  
35 by azeotropical distillation with an organic solvent

defined as above, and the resulting aqueous solution is lyophilized.

If the aqueous base employed in the elution is unvolatile, it may be necessary to neutralize and desalt the concentrate before precipitation or freeze-drying.

A convenient desalting procedure includes applying the antibiotic containing aqueous solution to a silanized silica gel column, washing with distilled water and eluting with a mixture of a polar water-miscible solvent and water.

Representative examples of polar water-miscible solvents are: water-soluble alcohols, (such as methanol, ethanol, iso-propanol, n-butanol), acetone, acetonitrile, lower alkyl alkanoates (such as ethyl acetate), tetrahydrofuran, dioxane and dimethylformamide and mixtures thereof; the preferred polar water-miscible solvent being acetonitrile.

Alternatively, desalting may be carried out by applying the antibiotic containing solution to the above described affinity column, washing with distilled water and eluting with a volatile aqueous base as described above for the elution of the affinity chromatography. The product so obtained is antibiotic A 40926 complex.

If necessary, it may be further purified or subjected as such to the separation of its factors A, B, B<sub>0</sub>, PA and PB.

A convenient procedure to obtain a pure antibiotic A 40926 complex is represented by a further purification of the complex as obtained above on an affinity chromatography column. The same stationary phase as above (immobilized D-Alanyl-D-Alanine) is generally used and the desired antibiotic substance is eluted by



following the affinity chromatography procedure on immobilized D-Alanyl-D-Alanine described above.

A preferred immobilized D-Alanyl-D-Alanine is Sepharose- $\epsilon$ -aminocaproyl-D-Alanyl-D-Alanine, a preferred  
5 equilibrating mixture is 0.16% (w/v) ammonia containing 2M NaCl adjusted to pH 7-8, a preferred rinsing solution is 0.16% (w/v) ammonia containing 2M NaCl adjusted to pH 8-9.5, a preferred eluting mixture is 0.16% (w/v) ammonia containing 2M NaCl adjusted to pH 10.5-12 and a  
10 most preferred eluting mixture is the above mixture adjusted to pH 11.5.

The antibiotic A 40926 factors, namely antibiotic A 40926 factor A, antibiotic A 40926 factor B,  
15 antibiotic A 40926 factor B<sub>0</sub>, antibiotic A 40926 factor PA and antibiotic A 40926 factor PB are isolated from an aqueous solution of antibiotic A 40926 complex by column chromatography and preferably by reverse-phase column chromatography. The preferred stationary phase in the  
20 case of reverse-phase column chromatography is silanized silica gel. Good results may be obtained however also with column chromatography on non-functionalized polystyrene and acrylic resins such as those sold under the trade names Amberlite XAD-2, XAD-4, XAD-7 and XAD-8  
25 (Rohm and Haas) or Diaion HP 20 (Mitsubishi).

In case the reverse-phase purification step is accomplished by means of a silanized silica gel as the stationary phase, the column is preferably  
pre-equilibrated with a buffered aqueous solution at pH  
30 between 4 and 9 and preferably between 5.5-6.5 and then eluted with a linear gradient of a polar water-miscible solvent in the same buffered solution. Representative examples of polar water-miscible solvents are:  
water-soluble alcohols, (such as methanol, ethanol,  
35 iso-propanol, n-butanol), acetone, acetonitrile,

tetrahydrofuran, dioxane and dimethylformamide and mixtures thereof; the preferred polar water-miscible solvent being acetonitrile.

- 5 The eluted fractions are assayed for their antibiotic content by means of the usual bioassays, such as paper-disc or agar-diffusion assays, on susceptible microorganisms. Examples of susceptible organisms are Bacillus subtilis and S. aureus.
- 10 The chromatography is also conveniently monitored by TLC or HPLC techniques.

- A preferred HPLC technique is represented by a reverse-phase HPLC using a column of porous and spheric particles of silanized silica gel functionalized with
- 15 C-18 alkyl groups having a diameter preferably of 5 micrometers (such as 5  $\mu$ m Ultrasphere<sup>®</sup> ODS Altex; Beckman Co.), a pre-column which is a silica gel functionalized with C-18 alkyl groups (such as RP 18 Brownlee Labs) and an eluent which is a linear gradient
- 20 mixture of a polar water miscible solvent, such as one of those described above, in an aqueous buffered solution.

- Preferably this solution is adjusted to pH 5-7. A most preferred eluent is represented by a linear gradient
- 25 from 5 to 60% of eluent B in eluent A wherein eluent A is a mixture of acetonitrile/aqueous buffer, pH 5-7, 10:90 and eluent B is a mixture of acetonitrile/aqueous buffer, pH 5-7, 70:30. As known in the art, many substances can be used as internal standards. A very
- 30 convenient one is, in this case, Teicoplanin A<sub>2</sub> component 2 (Gruppo Lepetit S.p.A.) which has a retention time close to the compounds of the invention in this HPLC system. This standard substance is known and has been described in GB-A- 2121401.
- 35

Fractions with a similar antibiotic content are pooled and desalted as described above to give essentially pure antibiotic A 40926 factor A, factor B, factor B<sub>0</sub>, factor PA, and factor PB.

5

Essentially pure antibiotic A 40926 factor A and antibiotic A 40926 factor B, antibiotic A 40926 factor B<sub>0</sub>, antibiotic A 40926 factor PA and antibiotic A 40926 factor PB are obtained from those fractions  
10 containing them by a variety of known techniques such as lyophilization, precipitation by non-solvents or precipitation by changing the pH of the aqueous solution.

A convenient procedure includes adding a solvent capable  
15 of forming azeotropic mixtures with water, removing water by azeotropic distillation and then collecting by filtration the precipitate obtained after addition of a non-solvent like those described above.

20 Antibiotic A 40926 factors PA and PB, at least under certain fermentation conditions, are the main antibiotic products of the A 40926 producing microorganism. Antibiotic A 40926 factors A and B are mainly transformation products of antibiotic A 40926 factor PA  
25 and factor PB respectively, and are often already present in the fermentation broth.

It has been found in fact that antibiotic A 40926 factor PA can be transformed into antibiotic A 40926 factor A and antibiotic A 40926 factor PB can be transformed into  
30 antibiotic A 40926 factor B under basic conditions. For instance, antibiotic A 40926 factor PA and antibiotic A 40926 factor PB are transformed into antibiotic A 40926 factor A and factor B respectively, by treatment  
35 with 0.5-10% aqueous ammonia or other nucleophilic base

such as an organic amine at room temperature for 8-24 hours.

As a consequence, when the fermentation broth, or an antibiotic A 40926 containing extract or concentrate thereof, is allowed to stand for a certain time under basic conditions (e.g. aqueous solution of a nucleophilic base, at a pH >9 overnight,) an antibiotic A 40926 complex will be obtained which is enriched in antibiotic A 40926 factor A and factor B. If the period of exposure of the fermentation broth, extract or concentrate thereof, to a basic environment is short, an antibiotic A 40926 complex is obtained which is enriched in antibiotic A 40926 factor PA and factor PB.

A preferred procedure to obtain an antibiotic A 40926 complex enriched in factor A and factor B includes therefore leaving a solution of antibiotic A 40926 complex (which contains mainly antibiotic A 40926 factors PA and PB) at room temperature for 8-12 h in an aqueous nucleophilic base, such as aqueous ammonia, and then isolating the desired antibiotic complex as described above.

Examples of A 40926 containing solutions are fermentation broths, extracts and affinity chromatography eluted fractions.

Pure antibiotic A 40926 may be obtained by further purifying the crude complex by affinity chromatography as described above.

The product so obtained which possesses biological and physico-chemical properties derivable from the pure factors thereof, will be referred to in the examples as antibiotic A 40926 complex AB.

A preferred procedure to enrich in factors PA and PB an antibiotic A 40926 complex preparation, includes rapidly neutralizing the affinity chromatography eluted fractions with an acid, preferably a mineral acid such as sulfuric or hydrochloric acid.

The isolation of pure antibiotic A 40926 factors PA and PB from this complex can be achieved according to one of the above reported procedures.

10

A preferred procedure includes reverse-phase liquid chromatography, preferably in stainless steel columns under moderate pressure (5-50 bar) or at high pressure (100-200 bar) The solid phase may be a silanized silica gel with a hydrocarbon phase at (2-18) carbon atoms (most preferably C 18) or phenyl group, and the eluent is a mixture of a polar water-miscible solvent as defined above and an aqueous buffer at a pH compatible with the resin (preferably pH 4-8).

20 The elution is monitored as usual, the fractions having homogeneous antibiotic content are pooled and treated as described above to isolate the pure compounds having the characteristics reported below.

25 Sophisticated HPLC analysis has shown that antibiotic A 40926 factor B actually is a mixture of two factors denominated factor B<sub>0</sub> and factor B<sub>1</sub>.

Antibiotic A 40926 factor B<sub>0</sub>, which accounts for approximately 90% of antibiotic A 40926 factor B, is the factor that has R<sub>t</sub> of 1.22 relative to Teicoplanin A<sub>2</sub> component 2 in the system described below under point D of the physico-chemical characteristics of factor B, while factor B<sub>1</sub>, which accounts for approximately 10% of antibiotic A 40926 factor B, is that with relative R<sub>t</sub> of 1.27 in the same system.

Pure antibiotic A 40926 factor B<sub>0</sub> is obtained by further purification of antibiotic A 40926 factor B for instance by repeating the reverse-phase chromatography procedure used for its isolation.

- 5 The physico-chemical and biological properties of antibiotic A 40926 factor B<sub>0</sub> are substantially identical to those of antibiotic A 40926 factor B except that at the HPLC analysis in a system like the above cited one, it has only one peak (R<sub>t</sub> 1.22 relative to Teicoplanin  
10 A<sub>2</sub> component 2 in the described HPLC system).

- Because of the above outlined similarities between antibiotic A 40926 factor B and antibiotic A 40926 factor B<sub>0</sub> in the present disclosure and claims the  
15 reference to the biological properties of antibiotic A 40926 factor B is to be understood as referring also to antibiotic A 40926 factor B<sub>0</sub> which is the main component (about 90%) of antibiotic A 40926 factor B and mainly contributes to its biological properties.

20

- Alternatively, the antibiotic substances of the invention may be isolated from the fermentation broth or further purified by means of strong or weak anion resins  
25 including functionalized polystyrene, acrylic or polydextrane matrices. Examples of weak anion exchange resins are those sold under the following trade-names: Dowex MWA-1 or WGR (Dow Chemical), Amberlite IRA-73 (Rohm and Haas), DEAE-Sephadex (Pharmacia). Examples of  
30 strong anion exchange resins which may be used according to invention include those sold under the following trade names: Dowex MSA-1, SBR, SBR-P (Dow Chemical), Amberlite IR-904 (Rohm and Haas) and QAE-Sephadex (Pharmacia).

- The elution of the antibiotic substances of the invention from these resins is conducted by means of linear gradient mixtures of aqueous solution of electrolytes, such as sodium or potassium hydrochlorides, in water or mixtures of water and an organic water-miscible solvent such as a lower alcohol (e. g. (C<sub>1</sub>-C<sub>4</sub>)alkanol) or lower alkyl ketones (e.g. acetone).
- 5
- 10 As already said, the antibiotic substances of the invention possess acid and basic functions and can form salts according to conventional procedures. Representative and suitable acid addition salts of the compounds of formula I include those salts formed by
- 15 standard reaction with both organic and inorganic acids such as, for example, hydrochloric, hydrobromic, sulfuric, phosphoric, acetic trifluoroacetic, trichloroacetic, succinic, citric, ascorbic, lactic, maleic, fumaric, palmitic, cholic, pamoic, mucic,
- 20 glutamic, camphoric, glutaric, glycolic, phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic, cinnamic and the like acids.
- Representative examples of these bases are:
- 25 alkali metal or alkaline-earth metal hydroxide such sodium, potassium, and calcium, hydroxide; ammonia and organic amines aliphatic, alicyclic or aromatic such as methylamine, dimethylamine, trimethylamine, and picoline.
- 30 The transformation of the free amino or non-salt compounds of the invention into the corresponding addition salts, and the reverse, i.e. the transformation of an addition salt of a compound of the invention into the non-salt or free amino form, are within the ordinary

technical skill and are encompassed by the present invention.

For instance, a compound of the invention can be transformed into the corresponding acid or base

5 addition-salt by dissolving the non-salt form in an aqueous solvent and adding a slight molar excess of the selected acid or base. The resulting solution or suspension is then lyophilized to recover the desired salt.

10 In case the final salt is insoluble in a solvent where the non-salt form is soluble it is recovered by filtration from the organic solution of the non-salt form after addition of the stoichometric amount or a slight molar excess of the selected acid or base.

15 The non-salt form can be prepared from a corresponding acid or base salt dissolved in an aqueous solvent which is then neutralized to free the non-salt form.

When following the neutralization desalting is necessary, a common desalting procedure may be employed.

20 For example, column chromatography on silanized silica gel, non-functionalized polystyrene, acrylic and controlled pore polydextrane resins (such as Sephadex LH 20) or activated carbon may be conveniently used. After eluting the undesired salts with an aqueous solution,  
25 the desired product is eluted by means of a linear gradient or a step-gradient of a mixture of water and a polar or apolar organic solvent, such as acetonitrile/water from 50:50 to about 100% acetonitrile.

30

As it is known in the art, the salt formation either with pharmaceutically acceptable acids (bases) or non-pharmaceutically acceptable acids (bases) may be used as a convenient purification technique. After

35 formation and isolation, the salt form of an A 40926



antibiotic can be transformed into the corresponding non-salt or into a pharmaceutically acceptable salt.

In some instances, the base addition salt of a compound of the invention is more soluble in water and hydrophilic solvents.

Physico-chemical characteristics of antibiotic A 40926  
factor A

A) ultraviolet absorption spectrum, which is shown in Figure 1 of the accompanying drawings, and exhibits the following absorption maxima:

|  | $\lambda$ max (nm)    |
|--|-----------------------|
| a) 0.1 N HCl                           | 281                   |
| b) phosphate buffer pH 7.38            | 281<br>300 (shoulder) |
| c) 0.1 N sodium or potassium hydroxide | 300                   |
| d) methanol                            | 282                   |
| e) phosphate buffer pH 9.0             | 282<br>300 (shoulder) |

B) infrared absorption spectrum which is shown in Figure 2 of the accompanying drawings and exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):

3700-3100, 3100-2800 (nujol); 1655; 1620-1560;  
1510; 1480-1410 (nujol); 1375 (nujol); 1320-1250;  
1250-1190; 1100-950; 845; 810; 720 (nujol).

C)  $^1\text{H}$ -NMR spectrum which is shown in Figure 3 and exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO  $d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$  = ppm):  
 $\delta$  0.86 (t's, 6H); 1.21 ( $\sim$ 11H); 1.43 (2H);  
2.01 (2H); 2.31-2.34 (3H); 4-6.2 ( $\sim$ 16H);  
6.2-8 ( $\sim$ 23H); 8.44, 9.22, 9.66 (broad bands; mobile protons)  
2.5-4: interference from  $\text{H}_2\text{O}$  peaks.

D) retention-time ( $R_t$ ) of 1.22 relative to Teicoplanin  $A_2$  component 2 ( $R_t$  = 20.3 min), when analyzed by reverse phase HPLC under the following conditions:

column: Ultrasphere ODS (5  $\mu\text{m}$ ) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu\text{m}$ )

eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

U.V. detector: 254 nm

- 5      internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

Under the same conditions the retention time  
relative to Testosterone (Roussel Uclaf) is 0.60.

10

- E) elemental analysis, after the sample has been  
previously dried at about 140°C under inert  
atmosphere ( $\Delta w$  4.6%) which indicates the following  
approximate percentage composition (average):  
15 carbon 55.82%; hydrogen 5.17%; nitrogen 6.31%;  
chlorine (total) 4.24%; chlorine (ionic) 0.37%.  
Inorganic residue at 900°C in the air: 1.2%.

- F) acid-base titration profile in 2-methoxyethanol  
20 (MCS):water, 4:1 upon titration with KOH after  
addition of an excess of HCl which indicates four  
ionizable functions having the following  $pK_{MCS}$ :  
4.6, 5.6, 7.2, 9.2.

- 25 G)  $R_f$  value of 0.24 and a  $R_f$  value relative to  
Teicoplanin A<sub>2</sub> component 2 of 0.70 in the following  
chromatographic system:

|  |    |
|--|----|
| 5% (w/v) aqueous Na <sub>2</sub> SO <sub>4</sub> | 70 |
| acetonitrile                                     | 30 |

30

using silanized silica gel 60 F<sub>254</sub> Merck plates  
(layer thickness 0.25 mm)

Visualization:

- U.V. light
- 5 - Yellow color with Pauly Reagent, i.e. diazotized  
sulfanilic acid (J. Chromatog. 20, 171 (1965),  
Z. Physiol. Chem. 292, 99, (1953))
- Bioautography using B. subtilis ATCC 6633 on  
minimal Davis medium.

10

- H) MW of about 1716 desumed from a FAB-MS spectrum  
showing the M+H<sup>+</sup> peak at 1717.

15

Physico-chemical characteristics of antibiotic A 40926  
factor B

20

- A) ultraviolet absorption spectrum, which is shown in  
Figure 4 of the accompanying drawings, and exhibits  
the following absorption maxima:

|    |  | $\lambda$ max (nm)    |
|----|--|-----------------------|
| 5  | a) 0.1 N HCl                           | 282                   |
|    | b) phosphate buffer pH 7.38            | 281<br>300 (shoulder) |
| 10 | c) 0.1 N sodium or potassium hydroxide | 300                   |
|    | d) phosphate buffer pH 9.0             | 283<br>300 (shoulder) |
| 15 | e) methanol                            | 282                   |

B) infrared absorption spectrum which is shown in Figure 5 of the accompanying drawings and exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):  
 3700-3080, 3080-2700 (nujol); 1720-1625; 1625-1560; 1505; 1460 (nujol); 1375 (nujol); 1295; 1230; 1210; 1150; 1100-1040; 1030; 1015; 970; 890; 840; 810; 720 (nujol).

C)  $^1\text{H}$ -NMR spectrum shown in Figure 6 exhibits the following groups of signals (in ppm) in the 270 MHz  $^1\text{H}$ -NMR recorded in DMSO  $\text{d}_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta = \text{ppm}$ ):  
 $\delta$  0.85 (d, isopropyl  $\text{CH}_3$ 's); 1.15 ( $\sim 13\text{H}$ ); 1.44 ( $\sim 2\text{H}$ ); 2.02 (2H); 2.32-2.35 (3H); 4-6.1 ( $\sim 16\text{H}$ ); 6.1-8 ( $\sim 23\text{H}$ ); 8.52, 9.30, 9.68 (broad bands; mobile protons)  
 2.5-4 interference from  $\text{H}_2\text{O}$  peaks.

- D) Retention times ( $R_t$ ) of 1.22 and 1.27 relative to Teicoplanin A<sub>2</sub> component 2 ( $R_t$  = 20.3 min) when analyzed by reverse phase HPLC under the following conditions:

5

column: Ultrasphere ODS (5  $\mu$ m) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu$ m)

10

eluent A: CH<sub>3</sub>CN 10% } adjusted at  
(2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 90% } pH 6.0

15

eluent B: CH<sub>3</sub>CN 70% } adjusted at  
(2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

20

flow rate: 1.8 ml/min

U.V. detector: 254 nm

25

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

30

- E) elemental analysis, after the sample has been previously dried at about 140°C under inert atmosphere ( $\Delta w$  9.6%) indicates the following approximate percentage composition (average): carbon 54.09%; hydrogen 5.13%; nitrogen 6.34%; chlorine (total) 4.12%; chlorine (ionic) 0.39%; Inorganic residue at 900°C in the air: 5%.

- F) acid base titration profile in 2-methoxyethanol (MCS):water, 4:1 upon titration with KOH after addition of an excess of HCl (pH 2.7) which indicates four ionizable functions having the following  $pK_{MCS}$ : 4.5, 5.6, 7.2, 9.2.
- G)  $R_f$  value of 0.21 and a  $R_f$  value relative to Teicoplanin A<sub>2</sub> component 2 of 0.53 in the following chromatographic system:
- |  |    |
|--|----|
| 5% (w/v) aqueous Na <sub>2</sub> SO <sub>4</sub> | 70 |
| acetonitrile                                     | 30 |
- using silanized silica gel 60 F<sub>254</sub> Merck plates (layer thickness 0.25 mm)
- Visualization:
- U.V. light
  - Yellow color with Pauly Reagent, i.e. diazotized sulfanilic acid (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
  - Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.
- H) MW of about 1730 desumed from a FAB-MS spectrum showing the  $M+H^+$  peak at 1731.

Physico-chemical characteristics of antibiotic A 40926  
factor B<sub>0</sub>

- A) ultraviolet absorption spectrum which is shown in Figure 4 of the accompanying drawing and exhibits the following absorption maxima:

|    |  | $\lambda$ max (nm)    |
|----|--|-----------------------|
| 5  | a) 0.1 N HCl                           | 282                   |
|    | b) phosphate buffer pH 7.38            | 281<br>300 (shoulder) |
| 10 | c) 0.1 N sodium or potassium hydroxide | 300                   |
|    | d) phosphate buffer pH 9.0             | 283<br>300 (shoulder) |
| 15 | e) methanol                            | 282                   |

B) infrared absorption spectrum which shown in Figure 5 of the accompanying drawings exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):  
 3700-3080, 3080-2700 (nujol); 1720-1625; 1625-1560; 1505; 1460 (nujol); 1375 (nujol); 1295; 1230; 1210; 1150; 1100-1040; 1030; 1015; 970; 890; 840; 810; 720 (nujol).

C)  $^1\text{H}$ -NMR spectrum which shown in Figure 6 exhibits the following groups of signals (in ppm) in the 270 MHz  $^1\text{H}$ -NMR recorded in DMSO  $d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$ = ppm):  
 0.85 (d, isopropyl  $\text{CH}_3$ 's); 1.15 ( $\sim 13\text{H}$ ); 1.44 ( $\sim 2\text{H}$ ); 2.02 (2H); 2.32-2.35 (3H); 4-6.1 ( $\sim 16\text{H}$ ); 6.1-8 ( $\sim 23\text{H}$ ); 8.52, 9.30, 9.68 (broad bands; mobile protons)  
 2.5-4 interference from  $\text{H}_2\text{O}$  peaks.



5 column: Ultrasphere ODS (5  $\mu$ m) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

10      eluent A: CH<sub>3</sub>CN                          10% } adjusted at  
                      (2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O     90% } pH 6.0

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

flow rate: 1.8 ml/min

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

25 E) elemental analysis, after the sample has been  
previously dried at about 140°C under inert  
atmosphere ( $\Delta w$  9.6%) indicates the following  
approximate percentage composition (average):  
30 carbon 54.09%; hydrogen 5.13%; nitrogen 6.34%;  
chlorine (total) 4.12%; chlorine (ionic) 0.39%.  
Inorganic residue at 900°C in the air: 5%.

F) acid base titration profile in 2-methoxyethanol (MCS):water, 4:1 upon titration with KOH after addition of an excess of HCl indicates four ionizable functions having the following  $pK_{MCS}$ :

5 4.5, 5.6, 7.2, 9.2.

G)  $R_f$  value of 0.21 and a  $R_f$  value relative to Teicoplanin  $A_2$  component 2 of 0.53 in the following chromatographic system:

|    |                             |    |
|----|-----------------------------|----|
| 10 | 5% (w/v) aqueous $Na_2SO_4$ | 70 |
|    | acetonitrile                | 30 |

using silanized silica gel 60  $F_{254}$  Merck plates (layer thickness 0.25 mm)

15 Visualization:

- U.V. light
- Yellow color with Pauly Reagent, i.e. diazotized sulfanilic acid (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- 20 - Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.

H) MW of about 1730 desumed from a FAB-MS spectrum showing the  $M+H^+$  peak at 1731.

25

Physico-chemical characteristics of antibiotic a 40926 factor PA

30 A) ultraviolet absorption spectrum, shown in Figure 7a and b, and spectrum of the accompanying drawings, exhibits the following absorption maxima:

$\lambda$  max (nm)

---

|    |                              |                |
|----|------------------------------|----------------|
| 5  | a) 0.1 N HCl                 | 282            |
|    | b) 0.1 N potassium hydroxide | 300            |
|    | c) phosphate buffer pH 7.38  | 282            |
| 10 |                              | 300 (shoulder) |
|    | d) phosphate buffer pH 9.0   | 283            |
|    |                              | 300 (shoulder) |

---

15

B) infrared absorption spectrum shown in Figure 8 of the accompanying drawings exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):

20 3700-3100, 3000-2800 (nujol); 1760-1710; 1655; 1620-1550; 1505; 1460 (nujol); 1375 (nujol); 1260, 1250-950; 845; 805; 720 (nujol)

25 C)  $^1\text{H}$ -NMR spectrum which is shown in Figure 9 exhibits the following groups of signals (in ppm) in the 270 MHz  $^1\text{H}$ -NMR recorded in DMSO  $d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$ = ppm):

0.86, d's ( $\text{CH}_3$ ); 1.15-1.22, m ( $\text{CH}_2$ )<sub>n</sub>; 1.41, m ( $\text{CH}_2$ ); 2.01, s ( $\text{CH}_3$ ); 2.01, m ( $\text{CH}_2$ ); 2.28, s ( $\text{N-CH}_3$ ); 4.26-5.96, br (peptidic and aromatic CH's); 6.33-7.73 br (aromatic CH's and peptidic NH's).

br = broad

d = doublet

dd = doublet of doublets

m = multiplet

s = singlet

t = triplet

D) retention-time ( $R_t$ ) of 1.15 relative to Teicoplanin A<sub>2</sub> component 2 ( $R_t$  = 20.3 min) when analyzed by reverse phase HPLC under the following conditions:

column: Ultrasphere ODS (5  $\mu\text{m}$ ) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu\text{m}$ )

eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

elution: linear gradient from 5% to 60% of eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

U.V. detector: 254 nm

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

- 5 E) R<sub>f</sub> value relative to Teicoplanin A<sub>2</sub> component 2 of  
0.62 in the following chromatographic system:  
5% (w/v) aqueous Na<sub>2</sub>SO<sub>4</sub> 70  
acetonitrile 30

10 using silanized silica gel 60 F<sub>254</sub> Merck plates  
(layer thickness 0.25 mm)

Visualization:

- U.V. light  
- Yellow color with Pauly Reagent, i.e. diazotized  
sulfanilic acid (J. Chromatog. 20, 171 (1965),  
15 Z. Physiol. Chem. 292, 99, (1953))  
- Bioautography using B. subtilis ATCC 6633 on  
minimal Davis medium.

- 20 F) MW of about 1758 desumed from a FAB-MS spectrum  
showing a cluster of peaks having the most intense  
peak at 1761. The operative conditions of the  
FAB-MS analysis were the following:

25 Instrument: VG Mod ZAB SE equipped with  
FAB gun Ion Tech

30 Conditions: Positive FAB, Xe  
Accelerating voltage, 8KV  
Matrix: Thioglycerol-glycerol  
1/1 (v/v).

Physico-chemical characteristics of antibiotic a 40926  
factor PB

- 5 A) ultraviolet absorption spectrum, shown in Figure 10  
 of the accompanying drawings, exhibits the  
 following absorption maxima:

|    |                              | $\lambda$ max (nm)    |
|----|------------------------------|-----------------------|
| 10 |                              |                       |
|    | a) 0.1 N HCl                 | 282                   |
|    | b) 0.1 N potassium hydroxide | 300                   |
| 15 | c) phosphate buffer pH 7.38  | 282<br>300 (shoulder) |
|    | d) phosphate buffer pH 9.0   | 282<br>300 (shoulder) |
| 20 |                              |                       |

- 25 B) infrared absorption spectrum which shown in Figure  
 11 of the accompanying drawings exhibits the  
 following absorption maxima ( $\text{cm}^{-1}$ ):

3700-3100, 3000-2800 (nujol); 1760-1710; 1655;  
 1620-1560; 1605; 1480-1420 (nujol); 1375 (nujol);  
 1320-1270; 1230-1190; 1150, 1120-920; 845; 810; 720;  
 30  
 (nujol)

C.1)  $^1\text{H}$ -NMR spectrum shown in Figure 12 exhibits the following groups of signals (in ppm) at the 270 MHz in  $\text{DMSO } d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$ = ppm)

5 multiplicity; (attribution):

0.84, d (isopropyl  $\text{CH}_3$ 's); 1.17, m  $(\text{CH}_2)_n$ ; 1.43, m  $(\text{CH}_2)$ , 1.99, s ( $\text{CH}_3$ ); 2.01, m ( $\text{CH}_2$ ); 2.31, s ( $\text{N-CH}_3$ ); 2.79, dd (C-H); 3.70, m (C-H); 4.06-6.02, br (peptidic and aromatic CH's); 6.45-7.74, br (aromatic CH's and peptidic NH's); 8.19-9.99, br (peptidic NH's and phenolic OH's)

C.2)  $^1\text{H}$ -NMR spectrum shown in Figure 13 exhibits the following groups of signals (in ppm) at the 270 MHz in  $\text{DMSO } d_6$  plus  $\text{CF}_3\text{COOD}$  using TMS as the internal standard (0.00 ppm), ( $\delta$ = ppm) multiplicity; (attribution):

15 0.84, d (isopropyl  $\text{CH}_3$ 's); 1.13, m  $(\text{CH}_2)_n$ ; 1.40, m  $(\text{CH}_2)$ ; 1.98, s ( $\text{CH}_3$ ); 2.00, m ( $\text{CH}_2$ ); 2.92, dd (C-H);  
20 3.29-3.71, m (sugar C-H's); 4.07-6.09, s and m (peptidic and aromatic CH's); 6.45-7.83, s and m (aromatic CH's and peptidic NH's); 8.17-10.38 (peptidic NH's, phenolic OH's).

25 D) retention times ( $R_t$ ) of 1.27 and 1.32 relative to Teicoplanin  $A_2$  component 2 ( $R_t$  = 20.3 min) when analyzed by reverse phase HPLC under the following conditions:

30 column: Ultrasphere ODS (5  $\mu\text{m}$ ) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu\text{m}$ )

eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
 (2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
 (2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

elution: linear gradient from 5% to 60% of  
 eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

U.V. detector: 254 nm

internal standard: Teicoplanin  $\text{A}_2$  component 2  
 (Gruppo Lepetit S.p.A.)

E)  $R_f$  value relative to Teicoplanin  $\text{A}_2$  component 2 of  
 0.53 in the following chromatographic system:

|   |    |
|---|----|
| 5% (w/v) aqueous $\text{Na}_2\text{SO}_4$ | 70 |
| acetonitrile                              | 30 |

using silanized silica gel 60  $\text{F}_{254}$  Merck plates  
 (layer thickness 0.25 mm)

Visualization:

- U.V. light
- Yellow color with Pauly Reagent, i.e. diazotized  
 sulfanilic acid (J. Chromatog. 20, 171 (1965),  
 Z. Physiol. Chem. 292, 99, (1953))
- Bioautography using B. subtilis ATCC 6633 on  
 minimal Davis medium.



F) MW of about 1772 desumed from a FAB-MS spectrum showing a cluster of peaks having the most intense peak at 1776. The operative conditions of the FAB-MS analysis were the following:

5

Instrument: VG Mod ZAB SE equipped with  
FAB gun Ion Tech

10 Conditions: Positive FAB, Xe  
Accelerating voltage, 8KV  
Matrix: Thioglycerol-glycerol  
1/1 (v/v).

15

The antibacterial activity of the compounds of the invention can be demonstrated in vitro by means of  
20 standard dilution tests on different microorganism cultures.

Culture media and growth conditions for MIC (minimal inhibitory concentration) determinations were as follows: Isosensitest broth (Oxoid), 24 h, for  
25 staphylococci, Strep. faecalis and Gram-negative bacteria ( Escherichia coli, Pseudomonas, Proteus);  
Todd-Hewitt broth (Difco), 24 h for other streptococcal species; GC base broth (Difco) + 1% Isovitalex (BBL), 48 h, CO<sub>2</sub>-enriched atmosphere for Neisseria gonorrhoeae; GB  
30 base agar (Difco) + 1% Isovitalex + 0.001% hemin, 24 h for Haemophilus influenzae; AC broth (Difco), 24 h, anaerobic atmosphere for Clostridium perfringens;  
Wilkins-Chalgren agar (ref: T.D. Wilkins & S. Chalgren, 1976, Antimicrob. Ag. Chemother. 10, 926); 48 h,  
35 anaerobic atmosphere for the other anaerobes ( C.

difficile, Propionibacterium acnes, Bacteroides fragilis); PPLO broth (Difco) + 10% horse serum + 1% glucose, 48 h for Mycoplasma gallisepticum; Yeast nitrogen base broth (Difco), 24 h for Candida albicans.

5 With the exception of C. albicans (30°C), incubation was at 37°C. Inocula were as follows: 1% (v/v) of a 48 h broth culture for M. gallisepticum; about  $10^4$ - $10^5$  colony-forming units/ml for other broth dilution MICs; about  $10^4$ - $10^5$  bacteria/spot (inoculated with a

10 multipoint inoculator) for agar dilution MICs ( H. influenzae, C. difficile, P. acnes, B. fragilis).

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TABLE IV

42

| <u>Strain</u>   | <u>Antibiotic A 40926</u> |                 |                  |                  |
|---|---------------------------|-----------------|------------------|------------------|
|   | <u>Factor A</u>           | <u>Factor B</u> | <u>Factor PA</u> | <u>Factor PB</u> |
|   | <u>M.I.C. (µg/ml)</u>     |                 |                  |                  |
| <u>Staph. aureus</u> ATCC 6538                          | 0.13                      | 0.13            |                  |                  |
| <u>Staph. aureus</u> Tour (L165) 10 <sup>4</sup> cfu/ml | 0.13                      | 0.13            | 0.5              | 1                |
| <u>Staph. aureus</u> Tour (L165) 10 <sup>6</sup> cfu/ml | 0.25                      | 0.13            | 2                | 2                |
| <u>Staph. epidermidis</u> ATCC 12228                    |                           |                 | 4                | 4                |
| <u>Strept. mitis</u> L 796 (clin. is.)                  |                           |                 | 0.06             | 0.06             |
| <u>Strep. pyogenes</u> C203                             | 0.063                     | 0.063           | 0.06             | 0.03             |
| <u>Strep. pneumoniae</u> UC41                           | 0.063                     | 0.063           | 0.06             | 0.06             |
| <u>Strep. faecalis</u> ATCC 7080                        | 0.13                      | 0.13            | 0.06             | 0.13             |
| <u>Clostr. perfringens</u> ISS 30543                    | 0.016                     | 0.016           | 0.008            | 0.008            |

TABLE IV (continued)

| Strain   | Antibiotic A 40926          |          |           |           |
|--|-----------------------------|----------|-----------|-----------|
|  | Factor A                    | Factor B | Factor PA | Factor PB |
|  | M.I.C. ( $\mu\text{g/ml}$ ) |          |           |           |
| <u>Clostr. difficile</u> ATCC 9689                 | 0.25                        | 0.13     | 0.25      | 0.13      |
| <u>Propion. acne</u> ATCC 6919                     | 0.03                        | 0.016    | 0.03      | 0.03      |
| <u>Neisseria gonorrhoeae</u> L 997 (clin. isol.) 1 |                             | 0.5      | 4         | 2         |
| <u>Haemophilus influenzae</u> ATCC 19418           | 64                          | 64       | 64        | 64        |
| <u>Proteus vulgaris</u> X19H ATCC 881              | >128                        | >128     | >128      | >128      |
| <u>Escherichia coli</u> SKF 12140                  | >128                        | >128     | >128      | >128      |
| <u>Pseudomonas aeruginosa</u> ATCC 10145           | >128                        | >128     | >128      | >128      |
| <u>Bacteroides fragilis</u> ATCC 23745             | 64                          | 64       | 64        | 64        |
| <u>Candida albicans</u> SKF 2270                   | >128                        | >128     |           |           |
| <u>Mycoplasma gallisepticum</u> Weybridge          | 64                          | 64       | 128       | 64        |

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TABLE V

Bactericidal effect on N. gonorrhoeae strains

| <u>N. gonorrhoeae strain</u> | <u>Antibiotic A 40926 complex</u> |           | <u>Spectinomycin</u> |           |
|------------------------------|-----------------------------------|-----------|----------------------|-----------|
|                              | MIC (48h)                         | MBC (24h) | MIC (48h)            | MBC (24h) |
| L 1001 *                     | 1                                 | 1         | 16                   | 32        |
| L 1004 *                     | 1                                 | 0.5       | 16                   | 32        |
| L 1007 (CTC 6820)            | 2                                 | 2         | 16                   | 16        |
| L 1596 **                    | 2                                 | 2         | >128                 | >128      |
| L 1601 ***                   | 1                                 | 1         | 16                   | 16        |
| L 1605 ***                   | 2                                 | 2         | 32                   | 32        |

MIC = minimal inhibitory concentration

MBC = minimal bactericidal concentration (lowest concentration at which 99.9% of the initial inoculum is killed within the indicated time)

\* = clinical isolate

\*\* = clinical isolate, spectinomycin-resistant

\*\*\* = clinical isolate, penicillin-resistant

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The antimicrobial activity of the compounds of the invention is confirmed also in in vivo experiments conducted essentially as described by R. Pallanza et al., J. Antimicrob. Chemother. 11, 419 (1983).

- 5 The experimental infection was induced in mice by intraperitoneally administering a suspension of S. pyogenes C 203. Inocula had been adjusted so that the untreated animals died of septicemia within 48 h. Animals were treated subcutaneously with the test
- 10 compound about 30 min after infection. The ED<sub>50</sub> value was calculated on the 10<sup>th</sup> day by the method of Spearman and Karber (D.J. Finney "Statistical Methods in Biological Assay", Griffin, page 524, 1952) on the basis of the percentage of survival at each dose.
- 15 In the above conditions the ED<sub>50</sub> for antibiotic A 40926 factor A was 0.47 mg/kg, for antibiotic A 40926 factor B was 0.33 mg/kg, for antibiotic A 40926 factor PA was 0.54 mg/kg and for antibiotic A 40926 factor PB 0.31 mg/kg.
- 20 The approximate acute toxicity in mice (i.p.) was evaluated according to methods known in the art. The approximate LD<sub>50</sub> of antibiotic A 40926 was found to be higher than 100 mg/kg in mice, when administered subcutaneosuly in mice.

25

Antibiotic A 40926 complex and its factors A, B, B<sub>0</sub>, PA and PB are active against gram-positive bacteria which are responsible for many widely diffused infections. Because of the increasing resistance of these pathogens to the usual therapeutic treatments, the need for new antibiotic substances is still great. As already stated, the antibiotic substances of the invention possess a good activity against Neisseria strains, such as Neisseria gonorrhoeae, while they are

practically inactive against other gram-negative bacteria.

5 It is known that the incidence of gonorrhea has risen steadily in the last 15-20 years.

The control of the infection is at present troublesome because of the high number of re-infections which is related also to the behaviour of infected individuals who do not take the necessary care in avoiding the  
10 transmission of the disease for the whole duration of the infection. On the other hand, there is an increasing resistance of the causative organism to the commonly used antibiotics so that new associations of antibiotics and longer treatments become necessary. Therefore there  
15 is an increasing need for new antibiotic substances which might be effective in curing gonococcal infections, and in particular Neisseria gonorrhoeae infections, with a limited number of administrations, or even with a single dose administration.

20

In general for antibacterial treatment antibiotic A 40926 complex, one of its factors A, B, B<sub>0</sub>, PA and PB as well as the non-toxic pharmaceutically acceptable salts thereof or mixture thereof, can be administered by  
25 different routes such as topically or parenterally. The parenteral administration is, in general, the preferred route of administration.

30 Compositions for injection may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain adjuvants such as suspending, stabilizing and/or dispersing agents.

Alternatively, the active ingredient may be in powder form for reconstitution at the time of delivery when a suitable vehicle, such as sterile water, is added  
35 thereto.

Depending on the route of administration, these compounds can be formulated into various dosage forms.

5 In some instances, it may be possible to formulate the compounds of the invention in enteric-coated dosage forms for oral administration which may be prepared as known in the art (see for instance "Remington's Pharmaceutical Sciences", fifteenth edition, Mack Publishing Company, Easton, Pennsylvania, USA, page 10 1614).

This could be especially the case when the absorption of the antimicrobial substance in the enteric tract is particularly desired while passing unaltered through the gastric tract.

15 The amount of active principle to be administered depends on various factors such as the size and condition of the subject to be treated, the route and frequency of administration, and the causative agent involved.

20 The antibiotic substances of the present invention, namely antibiotic A 40926 complex, its factors A, PA, B, B<sub>0</sub> and PB, and the physiologically acceptable salts thereof, are generally effective at a daily dosage of 25 between about 0.5 and 50 mg of active ingredient per kilo of patient body weight, optionally divided into 1 to 4 administrations per day.

Particularly desirable compositions are those prepared in dosage units containing from about 100 to about 30 5,000 mg per unit.

However, when used in the single-dose treatment of gonorrhea, higher minimum doses of antibiotic A 40926 complex, its factors A, PA, B, B<sub>0</sub> or PB, generally ranging between 0.5 and 50 mg/Kg, should be employed in



order to maintain an effective blood level of the drug over an extended period of time.

Furthermore, in the treatment of gonorrhea, a sustained-action parenteral dosage form is preferably employed. Sustained-action formulations can be prepared based on different mechanisms and methods, as known in the art.

A preferred method for preparing a sustained-action formulation containing antibiotic A 40926 complex, its factors A, B, B<sub>0</sub>, PA and PB involves the use of a water insoluble form of this antibiotic suspended in an aqueous or oily medium.

These forms, i.e. either as an insoluble salt or as the free acid, in fact, are released very slowly upon intramuscular injection, because of their low water-solubility, thus giving sustained blood levels of the antibiotic substance.

Preparation of pharmaceutical compositions:

A unit dosage form for intramuscular injection is prepared with 5 ml of sterile suspension USP containing 8% propylene glycol and 1,000 mg of antibiotic A 40926 factor A.

A unit dosage form for intramuscular injection is prepared with 5 ml of sterile suspension USP containing 8% propylene glycol and 500 mg of antibiotic A 40926 factor B.

A unit dosage form for intramuscular injection is prepared with 2,000 mg of antibiotic A 40926 factor B sodium salt suspended in 5 ml of sterile water for injection.

5

Furthermore, the antibiotics of the invention are useful for suppressing the growth of Clostridium difficile which causes pseudomembranous colitis in the intestine. The antibiotic could be used in the treatment of pseudomembranous colitis by the oral administration of an effective dose of the antibiotic or a pharmaceutically-acceptable salt thereof, prepared in a pharmaceutically-acceptable dosage form. For such use, the antibiotic can be administered in gelatin capsules or in liquid suspension.

Besides their activity as medicaments, the compounds of the present invention can be used as animal growth promoters.

For this purpose, one or more of the compounds of the invention is administered orally in a suitable feed. The exact concentration employed is that which is required to provide for the active agent in a growth promotant effective amount when normal amounts of feed are consumed.

The addition of the active compounds of the invention to animal feed is preferably accomplished by preparing an appropriate feed premix containing the active compounds in an effective amount and incorporating the premix into the complete ration.

Alternatively, an intermediate concentrate or feed supplement containing the active ingredient can be blended into the feed.

The way in which such feed premixes and complete rations can be prepared and administered are described in reference books (such as "Applied Animal Nutrition", W.H. Freedman and CO., S. Francisco, USA, 1969 or

5 "Livestock Feeds and Feeding" O and B books, Corvallis, Oregon, USA, 1977) and are incorporated herein by reference.

The following Examples further illustrate the invention

10 and, as such, should not be construed as limiting its scope.

Example 1: Fermentation of Actinomadura sp. ATCC 39727

15 A culture of antibiotic A 40926 producing strain (Actinomadura sp. ATCC 39727) is grown on oatmeal agar slant for 2-3 weeks at 28°C and used to inoculate a 500 ml Erlenmeyer flask containing 100 ml of medium composed of 0.5% meat extract, 0.5% autolyzed yeast,

20 0.5% peptone, 0.3% casein hydrolyzed, 2% glucose, 0.15% NaCl (pH 7.5 before sterilization).

The flask is incubated at 28°C on a rotary shaker at 200 rpm for about 72 h and then the culture is transferred to a fermentor containing 4 l of the above medium. This

25 culture is grown at 28°C for about 72 h with air-flow of about two liters per minute and stirring at about 900 rpm. Then, it is used to inoculate a 200 l fermentor of the same medium. This fermentor is aerated with 100 l per minute of sterile air and is stirred at 250 rpm at

30 about 28°C. The antibiotic production is monitored by the paper-disc agar diffusion method using B. subtilis on a minimal medium as the test organism. The maximum activity is obtained after 72-96 h.

Example 2: Recovery of antibiotic A 40926

- 5 A) The fermentation broth is cooled to 4°C, brought to pH 9.5 and stirred. After about 1 h it is filtered and the filtrate is adjusted to pH about 3.5 with an aqueous mineral acid. The mixture is stirred for 30 min at 4°C and then filtered with (Hyflo-FloMa<sup>®</sup>) filter aid. The clear filtrate is discharged and the filter cake is suspended in 10 deionized water, adjusted to pH about 8.5, stirred and then filtered. The recovered cake is subjected to the same procedure. The pooled filtrates contain antibiotic A 40926.
- 15 B) Swollen D-Ala-D-Ala-ε-aminocaproyl-Sepharose modified matrix (2 l) is added to the fermentation broth obtained according to Example 1 (after filtering it and bringing the pH of the clear 20 filtrate to about 8.5) or to the pooled filtrate obtained according to the above Example 2 A. After stirring overnight at room temperature, the resin is recovered by filtration and is washed sequentially with about 2 x 10 l of 0.45 mM 25 HCl-TRIS buffer pH 7.5 (TRIS = 2-amino-2-hydroxymethyl-1,3-propanediol) which contains 5% (w/v) NaCl and then with distilled water (4 x 20 l).
- 30 The A 40926 antibiotic is eluted from the resin with 1% (w/v) ammonia hydrate (2 x 20 l). The eluates are left overnight at room temperature and then concentrated to a small volume (about 2.5 l). Water is eliminated by azeotropical distillation with n-butanol. Petroleum ether is then added,

precipitating 3.4 g of crude antibiotic A 40926 complex.

5

Example 3:      Purification of antibiotic  
                  A 40926 complex AB

Crude antibiotic A 40926 complex obtained essentially  
10 following the procedure of the above Example 2, (750 mg;  
HPLC titre 70%) is dissolved in 400 ml of water,  
adjusted to pH 7.5 and filtered. The filtrate is then  
subjected to affinity chromatography on a  
D-Ala-D-Ala- $\epsilon$ -aminocaproyl-Sepharose column (50 ml of  
15 swollen resin; bed height = 5 cm). The column,  
equilibrated with 0.16% (w/v) ammonia containing 2 M  
NaCl adjusted to pH 7.5 with HCl, is developed  
sequentially with the following three buffer solutions:

20 buffer A: 0.16% (w/v) ammonia containing 2M NaCl adjusted  
to pH 7.5 with HCl, (2.6 column bed volumes);

buffer B: 0.16% (w/v) ammonia containing 2M NaCl  
adjusted to pH 9.5 with HCl (16 column bed  
25 volumes);

buffer C: 1% (w/v) aqueous ammonia pH 11.4 (2.6 column  
bed volumes).

30 Buffer C elutes antibiotic A 40926 complex AB in a  
single fraction. This eluted fraction is adjusted to  
pH 7.0 and reapplied to the same affinity column  
buffered with 10 mM TRIS-HCl pH 7.0. The column is  
washed with distilled water until desalting is complete.

The antibiotic is then eluted with 2 column bed volumes of 0.39% (w/v) aqueous ammonia pH 11.0.

The eluted fractions are concentrated to a small aqueous mixture and then freeze-dried. Pure antibiotic A 40926

5 complex AB (374 mg) is obtained.

Example 4:        Isolation of antibiotic A 40926  
                     factor A and B

10    A)    Antibiotic A 40926 complex as obtained according to  
         Example 2 (3.3 g) or antibiotic A 40926 complex AB  
         as obtained according to Example 3 (2.3 g) is  
         suspended in 0.5 l of water, stirred and then  
15       filtered. The clear filtrate is applied to a  
         silanized silica gel column (200 g; bed h 18 cm;  
         silanized Silicagel 60; 70-230 mesh, Merck Inc.)  
         pre-equilibrated with solution A (0.001 M aqueous  
         sodium EDTA containing 0.25% (w/v)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and  
         2.5% (w/v) NaCl adjusted to pH 6.0 with NaOH). The  
20       column is eluted with a linear gradient from 0% to  
         40% (v/v) of acetonitrile in solution A with a  
         total volume of about 7 l in about 48 h. Fractions  
         of about 15.5 ml are collected and assayed by  
         bioassay on Bacillus subtilis and analyzed by HPLC.  
25       Fractions having a similar antibiotic content are  
         pooled. Fractions No. 310-330 and No. 348-365  
         contained the antibiotic substances denominated,  
         respectively, A 40926 factor A and A 40926 factor B.  
         B.

30

B)    The pooled fractions containing the single A 40926  
         factors A and B are concentrated under reduced  
         pressure to remove acetonitrile, diluted with water  
         (about twice the volume of the initial solutions)  
35       and applied to a silanized silica gel column of the

type described above (volume of the swollen matrix: 50 ml; bed height of 15 cm). The column is washed with deionized water until desalting is complete and finally developed with acetonitrile/water 60:40 (v/v).

The eluted fractions are concentrated under reduced pressure and the residues are freeze-dried to obtain 134 mg of antibiotic A 40926 from the first group of eluted fractions (fractions 310-330 above) and 206 mg of A 40926 factor B from the second group of eluted fractions (fractions 348-365, above).

Example 5:        Isolation of antibiotic A 40926  
15                    factor PA and factor PB

By essentially following the procedure of Example 2A and the first steps of the procedure of Example 2B, the antibiotic linked to the resin is eluted with 1% (w/v) ammonia hydrate (2 x 20 l). The eluates are adjusted to pH 7.8 with sulfuric acid and concentrated to a small volume under vacuum by azeotropical distillation with n-butanol to obtain an aqueous concentrate which is then filtered on paper. The recovered filtrate contains antibiotic A 40926 factor PA, A 40926 factor PB and minor amounts of A 40926 factor A and factor B (HPLC). A sample (10 ml) of this aqueous concentrate containing about 50 mg/ml of pure antibiotic A 40926 complex (HPLC analysis) is filtered on 5 micrometer pore-size filter (Acrodisc®; Gelman Science Inc.) and then applied to a stainless steel column (diameter = 2 cm) containing 20 g of an octadecyl silyl reverse-phase silica gel (Lichrisorb RP 18, Merck Inc.; particle size 10 µm). The silica gel is then packed under moderate pressure (nominal pressure about 14 bar) in a stainless steel

column of a Chromatospac Modulprep apparatus (Yoben Yvon, France) and equilibrated with a mixture consisting of acetonitrile and 18 mM sodium phosphate buffer pH 6.0), 25:75 (v/v). The elution is carried out using the same solvent mixture used for the equilibration at a flow rate of about 10.5 ml/min. The eluate is monitored by bioassay on Bacillus subtilis and by HPLC.

Those fractions having similar antibiotic content are pooled and the homogeneous fractions of 5 chromatographic runs are concentrated to evaporate the organic solvent.

The resulting solution is diluted with aqueous 1M sodium chloride to twice the original volume and is applied to a silanized silica gel column (50 g; bed height 5 cm; 15 Silanized silica gel 60; Merck Inc.) equilibrated with water.

The column is washed with deionized water until desalting is complete (no AgCl precipitation in the eluates after addition of aqueous AgNO<sub>3</sub>) and then eluted with acetonitrile:water 1:1 (v/v). The eluates having similar antibiotic content (HPLC analysis) are pooled, concentrated to a small volume by azeotropic distillation with n-butanol to obtain an aqueous phase which is then freeze-dried. Yields:

25 antibiotic A 40926 factor PA : 55 mg  
antibiotic A 40926 factor PB : 51 mg  
antibiotic A 40926 factor A : 38 mg  
antibiotic A 40926 factor B<sub>0</sub> : 33 mg

30 Example 6: Alternative method for isolating antibiotic  
A 40926 factor B

The pooled concentrate of two preparations made according to Example 2 (the last step) is filtered and the filtrate is applied to a silanized silica gel



chromatographic column; (400 g; bed h 30 cm; Silicagel 60, 70-230 mesh, Merck Inc.) pre-equilibrated with water.

5 The column is rinsed with water (6 l) and the adsorbed antibiotic is eluted with acetonitrile/water according to the following sequence:

- 2.7 l 5% (v/v) acetonitrile in water
- 1.6 l 10% (v/v) acetonitrile in water
- 2.97 l 15% (v/v) acetonitrile in water
- 10 3.15 l 20% (v/v) acetonitrile in water

Fractions of about 18 ml are collected.

The activity of the eluted fractions is tested by paper-disc bioassay on susceptible microorganisms such as B. subtilis and analyzed by HPLC. Fractions with  
15 similar antibiotic content are pooled (fractions 472-526) and concentrated under reduced pressure. n-Butanol is added to this concentrate to azeotropically remove water. The butanolic solution which remains is in turn concentrated to a small volume to precipitate  
20 antibiotic A 40926 factor B (1.4 g). This product is washed with petroleum ether under stirring and collected by filtration (three times). Upon drying under vacuum 760 mg of A 40926 factor B are obtained.

25 By resubmitting antibiotic A 40926 factor B to the above column chromatography a product (540 mg) antibiotic A 40926 factor B<sub>0</sub> is obtained which has the same physico-chemical characteristics reported above for antibiotic A 40926 factor B except that it shows only a  
30 peak at HPLC analysis, namely the peak with retention time of 1.22 relative to Teicoplanin A<sub>2</sub> component 2.

Example 7: Transformation of antibiotic A 40926 factor PA and antibiotic A 40926 factor PB into antibiotic A 40926 factor A and factor B, respectively

5

Antibiotic A 40926 factor PA and antibiotic A 40926 factor PB (50 mg) are separately dissolved in 2.5 ml of aqueous 1% (w/v)  $\text{NH}_4\text{OH}$  and the resulting solutions are kept for about 24 h at room temperature with stirring. Antibiotic A 40926 factor A is obtained from the solution originally containing antibiotic A 40926 factor PA, and antibiotic A 40926 factor B is obtained from the solution originally containing antibiotic A 40926 factor PB by removing water by azeotropic distillation with n-butanol, precipitating with ethyl ether and collecting the precipitate by filtration (yield about 75%).

20

Preparation of D-Ala-D-Ala-Sepharose

Activated CH-Sepharose 4B (Pharmacia Fine Chemicals) (1g) is swollen for 15 minutes in 1 mM cold ice hydrochloric acid and washed with the same solution. The obtained gel (about 3 ml) is mixed with a solution of D-alanyl-D-alanine (30 mg) in 0.5 M sodium chloride and 0.1 M sodium bicarbonate buffer at pH 8. The mixture is rotated end-over-end for 1 hour at room temperature. After the coupling reaction is completed, the ligand excess is washed off with the buffer. The unlinked activated groups of the dextrane support are blocked by treating them with 1 M ethanolamine hydrochloride at pH 9 for 1 hour.

Then the Sephadex- $\epsilon$ -aminocaproyl-D-alanyl-D-alanine modified matrix is recovered by filtration and thoroughly washed alternatively with 0.5 M sodium chloride and 0.1 M sodium acetate pH 4, and with 0.5 M  
5 sodium chloride and 0.1 M tris(hydroxymethyl)amino- methane buffer pH 8. (four times).

CLAIMS

1. An antibiotic substance selected from antibiotic  
5 A 40926 complex, antibiotic A 40926 factor A, antibiotic  
A 40926 factor B, antibiotic A 40926 factor B<sub>0</sub>,  
antibiotic A 40926 factor PA, antibiotic A 40926 factor  
PB, a mixture thereof and the addition salts thereof  
which are characterized as following:

10

Antibiotic A 40926 factor A in the non-salt form:

- A) ultraviolet absorption spectrum which exhibits the  
following absorption maxima:

15

 $\lambda$  max (nm)

a) 0.1 N HCl

281

20

b) phosphate buffer pH 7.38

281

300 (shoulder)

c) 0.1 N sodium or potassium  
hydroxide

300

25

d) methanol

282

e) phosphate buffer pH 9.0

282

300 (shoulder)

30

- B) infrared absorption spectrum which exhibits the  
following absorption maxima (cm<sup>-1</sup>):

3700-3100, 3100-2800 (nujol); 1655; 1620-1560;

1510; 1480-1410 (nujol); 1375 (nujol); 1320-1250;

35

1250-1190; 1100-950; 845; 810; 720 (nujol).

C)  $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO  $d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$  = ppm):

5  $\delta$  0.86 (t's, 6H); 1.21 ( $\sim$ 11H); 1.43 (2H);  
2.01 (2H); 2.31-2.34 (3H); 4-6.2 ( $\sim$ 16H);  
6.2-8 ( $\sim$ 23H); 8.44, 9.22, 9.66 (broad bands; mobile protons)  
2.5-4: interference from  $\text{H}_2\text{O}$  peaks.

10

D) retention-time ( $R_t$ ) of 1.22 relative to Teicoplanin  $A_2$  component 2 ( $R_t$  = 20.3 min), when analyzed by reverse phase HPLC under the following conditions:

15 column: Ultrasphere ODS (5  $\mu\text{m}$ ) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu\text{m}$ )

20 eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

25

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

30

U.V. detector: 254 nm

internal standard: Teicoplanin  $A_2$  component 2  
(Gruppo Lepetit S.p.A.)

35

Under the same conditions the retention time relative to Testosterone (Roussel Uclaf) is 0.60.

- 5 E) elemental analysis, after the sample has been previously dried at about 140°C under inert atmosphere ( $\Delta w$  4.6%) which indicates the following approximate percentage composition (average): carbon 55.82%; hydrogen 5.17%; nitrogen 6.31%; chlorine (total) 4.24%; chlorine (ionic) 0.37%.  
10 Inorganic residue at 900°C in the air: 1.2%.
- F) acid-base titration profile in 2-methoxyethanol (MCS):water, 4:1 upon titration with KOH after addition of an excess of HCl which indicates four  
15 ionizable functions having the following  $pK_{MCS}$ : 4.6, 5.6, 7.2, 9.2.
- G)  $R_f$  value of 0.24 and a  $R_f$  value relative to Teicoplanin  $A_2$  component 2 of 0.70 in the following  
20 chromatographic system:  
5% (w/v) aqueous  $Na_2SO_4$  70  
acetonitrile 30
- using silanized silica gel 60 F<sub>254</sub> Merck plates  
25 (layer thickness 0.25 mm)  
Visualization:  
- U.V. light  
- Yellow color with Pauly Reagent, i.e. diazotized  
sulfanilic acid (J. Chromatog. 20, 171 (1965),  
30 Z. Physiol. Chem. 292, 99, (1953))  
- Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.
- H) MW of about 1716 desumed from a FAB-MS spectrum  
35 showing the  $M+H^+$  peak at 1717.

Antibiotic A 40926 factor B in the non-salt form:

A) ultraviolet absorption spectrum which exhibits the following absorption maxima:

|    |  |                    |
|----|--|--------------------|
| 5  |  | $\lambda$ max (nm) |
|    | a) 0.1 N HCl                           | 282                |
|    | b) phosphate buffer pH 7.38            | 281                |
| 10 |  | 300 (shoulder)     |
|    | c) 0.1 N sodium or potassium hydroxide | 300                |
| 15 | d) phosphate buffer pH 9.0             | 283                |
|    |  | 300 (shoulder)     |
|    | e) methanol                            | 282                |

20 B) infrared absorption spectrum which exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):  
 3700-3080, 3080-2700 (nujol); 1720-1625; 1625-1560;  
 1505; 1460 (nujol); 1375 (nujol); 1295; 1230; 1210;  
 1150; 1100-1040; 1030; 1015; 970; 890; 840; 810;  
 25 720 (nujol).

30 C)  $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) in the 270 MHz  $^1\text{H}$ -NMR recorded in DMSO  $d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$  = ppm):  
 $\delta$  0.85 (d, isopropyl  $\text{CH}_3$ 's); 1.15 ( $\sim$ 13H); 1.44 ( $\sim$ 2H); 2.02 (2H); 2.32-2.35 (3H); 4-6.1 ( $\sim$ 16H); 6.1-8 ( $\sim$ 23H); 8.52, 9.30, 9.68 (broad bands; mobile protons)  
 35 2.5-4 interference from  $\text{H}_2\text{O}$  peaks.

- D) Retention times ( $R_t$ ) of 1.22 and 1.27 relative to Teicoplanin A<sub>2</sub> component 2 ( $R_t$  = 20.3 min) when analyzed by reverse phase HPLC under the following conditions:

5

column: Ultrasphere ODS (5  $\mu$ m) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

10

pre-column: Brownlee Labs RP 18 (5  $\mu$ m)

eluent A: CH<sub>3</sub>CN 10% } adjusted at  
(2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 90% } pH 6.0

15

eluent B: CH<sub>3</sub>CN 70% } adjusted at  
(2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

20

flow rate: 1.8 ml/min

U.V. detector: 254 nm

25

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

30

- E) elemental analysis, after the sample has been previously dried at about 140°C under inert atmosphere ( $\Delta w$  9.6%) indicates the following approximate percentage composition (average):  
carbon 54.09%; hydrogen 5.13%; nitrogen 6.34%; chlorine (total) 4.12%; chlorine (ionic) 0.39%.  
Inorganic residue at 900°C in the air: 5%.



F) acid base titration profile in 2-methoxyethanol (MCS):water, 4:1 upon titration with KOH after addition of an excess of HCl (pH 2.7) which indicates four ionizable functions having the following  $pK_{MCS}$ : 4.5, 5.6, 7.2, 9.2.

G)  $R_f$  value of 0.21 and a  $R_f$  value relative to Teicoplanin A<sub>2</sub> component 2 of 0.53 in the following chromatographic system:

|  |    |
|--|----|
| 5% (w/v) aqueous Na <sub>2</sub> SO <sub>4</sub> | 70 |
| acetonitrile                                     | 30 |

using silanized silica gel 60 F<sub>254</sub> Merck plates (layer thickness 0.25 mm)

Visualization:

- U.V. light
- Yellow color with Pauly Reagent, i.e. diazotized sulfanilic acid (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.

H) MW of about 1730 desumed from a FAB-MS spectrum showing the  $M+H^+$  peak at 1731.

Antibiotic A 40926 factor B<sub>0</sub> in the non-salt form:

A) ultraviolet absorption spectrum which exhibits the following absorption maxima:

- |    |  | $\lambda$ max (nm)    |
|----|--|-----------------------|
|    | a) 0.1 N HCl   | 282                   |
| 5  | b) phosphate buffer pH 7.38  | 281<br>300 (shoulder) |
|    | c) 0.1 N sodium or potassium hydroxide   | 300                   |
| 10 | d) phosphate buffer pH 9.0   | 283<br>300 (shoulder) |
|    | e) methanol  | 282                   |
| 15 | B) infrared absorption spectrum which exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):   |                       |
|    | 3700-3080, 3080-2700 (nujol); 1720-1625; 1625-1560; 1505; 1460 (nujol); 1375 (nujol); 1295; 1230; 1210; 1150; 1100-1040; 1030; 1015; 970; 890; 840; 810; 720 (nujol).  |                       |
| 20 | C) $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) in the 270 MHz $^1\text{H}$ -NMR recorded in $\text{DMSO } d_6$ (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta = \text{ppm}$ ): |                       |
| 25 | $\delta$ 0.85 (d, isopropyl $\text{CH}_3$ 's); 1.15 ( $\sim 13\text{H}$ ); 1.44 ( $\sim 2\text{H}$ ); 2.02 (2H); 2.32-2.35 (3H); 4-6.1 ( $\sim 16\text{H}$ ); 6.1-8 ( $\sim 23\text{H}$ ); 8.52, 9.30, 9.68 (broad bands; mobile protons)                      |                       |
| 30 | 2.5-4 interference from $\text{H}_2\text{O}$ peaks.  |                       |
| 35 | D) Retention time ( $R_t$ ) of 1.22 relative to Teicoplanin $A_2$ component 2 ( $R_t = 20.3$ min) when analyzed by reverse phase HPLC under the following conditions:  |                       |

column: Ultrasphere ODS (5  $\mu$ m) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu$ m)

5

eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0

10

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

15

flow rate: 1.8 ml/min

U.V. detector: 254 nm

20

internal standard: Teicoplanin  $\text{A}_2$  component 2  
(Gruppo Lepetit S.p.A.)

25

- E) elemental analysis, after the sample has been previously dried at about 140°C under inert atmosphere ( $\Delta w$  9.6%) indicates the following approximate percentage composition (average):  
carbon 54.09%; hydrogen 5.13%; nitrogen 6.34%;  
chlorine (total) 4.12%; chlorine (ionic) 0.39%.  
Inorganic residue at 900°C in the air: 5%.

30

- F) acid base titration profile in 2-methoxyethanol (MCS):water, 4:1 upon titration with KOH after addition of an excess of HCl indicates four ionizable functions having the following  $\text{pK}_{\text{MCS}}$ :  
4.5, 5.6, 7.2, 9.2.

35

- G)  $R_f$  value of 0.21 and a  $R_f$  value relative to Teicoplanin A<sub>2</sub> component 2 of 0.53 in the following chromatographic system:

|   |  |    |
|---|--|----|
| 5 | 5% (w/v) aqueous Na <sub>2</sub> SO <sub>4</sub> | 70 |
|   | acetonitrile                                     | 30 |

using silanized silica gel 60 F<sub>254</sub> Merck plates  
(layer thickness 0.25 mm)

Visualization:

- 10 - U.V. light
- Yellow color with Pauly Reagent, i.e. diazotized sulfanilic acid (J. Chromatog. 20, 171 (1965), Z. Physiol. Chem. 292, 99, (1953))
- 15 - Bioautography using B. subtilis ATCC 6633 on minimal Davis medium.

- H) MW of about 1730 desumed from a FAB-MS spectrum showing the  $M+H^+$  peak at 1731.

20

Antibiotic A 40926 factor PA in the non-salt form:

- A) An ultraviolet absorption spectrum which exhibits the following absorption maxima:

|    |   | $\lambda$ max (nm)    |
|----|---|-----------------------|
| 5  | a) 0.1 N HCl  | 282                   |
|    | b) 0.1 N potassium hydroxide  | 300                   |
|    | c) phosphate buffer pH 7.38   | 282<br>300 (shoulder) |
| 10 | d) phosphate buffer pH 9.0  | 283<br>300 (shoulder) |
| 15 | B) infrared absorption spectrum which exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):<br>3700-3100, 3000-2800 (nujol); 1760-1710; 1655;<br>1620-1550; 1505; 1460 (nujol); 1375 (nujol); 1260,<br>1250-950; 845; 805; 720 (nujol)       |                       |
| 20 | C) $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) in the 270 MHz $^1\text{H}$ -NMR recorded in DMSO $\text{d}_6$ (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$ = ppm): |                       |

0.86, d's ( $\text{CH}_3$ ); 1.15-1.22, m ( $\text{CH}_2$ )<sub>n</sub>; 1.41, m  
( $\text{CH}_2$ ); 2.01, s ( $\text{CH}_3$ ); 2.01, m ( $\text{CH}_2$ ); 2.28, s  
(N- $\text{CH}_3$ ); 4.26-5.96, br (peptidic and aromatic  
CH's); 6.33-7.73 br (aromatic CH's and peptidic  
NH's).

br = broad

d = doublet

dd = doublet of doublets

m = multiplet

s = singlet

t = triplet

- 15 D) retention time ( $R_t$ ) of 1.15 relative to Teicoplanin  
A<sub>2</sub> component 2 ( $R_t$  = 20.3 min) when analyzed by  
reverse phase HPLC under the following conditions:

column: Ultrasphere ODS (5  $\mu\text{m}$ ) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu\text{m}$ )

eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

U.V. detector: 254 nm

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

- 5 E) R<sub>f</sub> value relative to Teicoplanin A<sub>2</sub> component 2 of  
0.62 in the following chromatographic system:  
5% (w/v) aqueous Na<sub>2</sub>SO<sub>4</sub> 70  
acetonitrile 30

10 using silanized silica gel 60 F<sub>254</sub> Merck plates  
(layer thickness 0.25 mm)

Visualization:

- 15 - U.V. light  
- Yellow color with Pauly Reagent, i.e. diazotized  
sulfanilic acid (J. Chromatog. 20, 171 (1965),  
Z. Physiol. Chem. 292, 99, (1953))  
- Bioautography using B. subtilis ATCC 6633 on  
minimal Davis medium.

- 20 F) MW of about 1758 desumed from a FAB-MS spectrum  
showing a cluster of peaks having the most intense  
peak at 1761. The operative conditions of the  
FAB-MS analysis were the following:

25 Instrument: VG Mod ZAB SE equipped with  
FAB gun Ion Tech

30 Conditions: Positive FAB, Xe  
Accelerating voltage, 8KV  
Matrix: Thioglycerol-glycerol  
1/1 (v/v).

Antibiotic A 40926 factor PB in the non-salt form:

- A) An ultraviolet absorption spectrum which exhibits the following absorption maxima:

|    |                              |                       |
|----|------------------------------|-----------------------|
| 5  |                              | $\lambda$ max (nm)    |
|    | a) 0.1 N HCl                 | 282                   |
| 10 | b) 0.1 N potassium hydroxide | 300                   |
|    | c) phosphate buffer pH 7.38  | 282<br>300 (shoulder) |
| 15 | d) phosphate buffer pH 9.0   | 282<br>300 (shoulder) |

- B) infrared absorption spectrum which exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):  
 3700-3100, 3000-2800 (nujol); 1760-1710; 1655;  
 1620-1560; 1605; 1480-1420 (nujol); 1375 (nujol);  
 1320-1270; 1230-1190; 1150, 1120-920; 845; 810; 720  
 (nujol)

25

- C.1)  $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) at the 270 MHz in  $\text{DMSO } d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta$  = ppm)  
 multiplicity; (attribution):

30



0.84, d (isopropyl CH<sub>3</sub>'s); 1.17, m (CH<sub>2</sub>)<sub>n</sub>; 1.43, m (CH<sub>2</sub>), 1.99, s (CH<sub>3</sub>); 2.01, m (CH<sub>2</sub>); 2.31, s (N-CH<sub>3</sub>); 2.79, dd (C-H); 3.70, m (C-H); 4.06-6.02, br (peptidic and aromatic CH's); 6.45-7.74, br (aromatic CH's and peptidic NH's); 8.19-9.99, br (peptidic NH's and phenolic OH's)

C.2) <sup>1</sup>H-NMR spectrum which exhibits the following groups of signals (in ppm) at the 270 MHz in DMSO d<sub>6</sub> plus CF<sub>3</sub>COOD using TMS as the internal standard (0.00 ppm), (δ = ppm) multiplicity; (attribution):

0.84, d (isopropyl CH<sub>3</sub>'s); 1.13, m (CH<sub>2</sub>)<sub>n</sub>; 1.40, m (CH<sub>2</sub>); 1.98, s (CH<sub>3</sub>); 2.00, m (CH<sub>2</sub>); 2.92, dd (C-H); 3.29-3.71, m (sugar C-H's); 4.07-6.09, s and m (peptidic and aromatic CH's); 6.45-7.83, s and m (aromatic CH's and peptidic NH's); 8.17-10.38 (peptidic NH's, phenolic OH's).

D) retention times (R<sub>t</sub>) of 1.27 and 1.32 relative to Teicoplanin A<sub>2</sub> component 2 (R<sub>t</sub> = 20.3 min) when analyzed by reverse phase HPLC under the following conditions:

column: Ultrasphere ODS (5 μm) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5 μm)

eluent A: CH<sub>3</sub>CN 10% } adjusted at  
(2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 90% } pH 6.0

eluent B: CH<sub>3</sub>CN 70% } adjusted at  
(2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

U.V. detector: 254 nm

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

E) R<sub>f</sub> value relative to Teicoplanin A<sub>2</sub> component 2 of  
0.53 in the following chromatographic system:

|  |    |
|--|----|
| 5% (w/v) aqueous Na <sub>2</sub> SO <sub>4</sub> | 70 |
| acetonitrile                                     | 30 |

using silanized silica gel 60 F<sub>254</sub> Merck plates  
(layer thickness 0.25 mm)

Visualization:

- U.V. light

- Yellow color with Pauly Reagent, i.e. diazotized  
sulfanilic acid (J. Chromatog. 20, 171 (1965),  
Z. Physiol. Chem. 292, 99, (1953))

- Bioautography using B. subtilis ATCC 6633 on  
minimal Davis medium.

F) MW of about 1772 desumed from a FAB-MS spectrum  
showing a cluster of peaks having the most intense  
peak at 1776. The operative conditions of the  
FAB-MS analysis were the following:

Instrument: VG Mod ZAB SE equipped with  
FAB gun Ion Tech

5        Conditions: Positive FAB, Xe  
Accelerating voltage, 8KV  
Matrix: Thioglycerol-glycerol  
1/1 (v/v).

10

2. A process for preparing a compound of claim 1 which comprises cultivating the strain Actinomadura sp. ATCC 39727, or an antibiotic A 40926-producing mutant or variant thereof, under submerged aerobic conditions in  
15 the presence of assimilable sources of carbon, nitrogen and inorganic salts, recovering and isolating said antibiotic from the fermentation broths and transforming it into the desired salt, if required.

20

3. A process as in claim 2 wherein the strain is cultivated at a temperature between 20°C and 40°C.

25

4. A process as in claim 2 wherein the temperature is between 24°C and 35°C.

30

5. A process as in claim 2 wherein the recovery and isolation of the antibiotic substances is obtained by submitting the filtered fermentation broth to an affinity chromatography on immobilized D-Alanyl-Alanine followed by partition, reverse-phase or ion-exchange chromatography.

35

6. A process according to claim 2 wherein the recovery of the antibiotic substances includes:

- 5 a) submitting the fermentation broth to affinity chromatography on immobilized D-Alanyl-D-Alanine
- b) rapidly neutralizing the pooled antibiotic containing eluted fractions and
- 10 c) optionally isolating the antibiotic A 40926 factors PA, PB, A, B and B<sub>0</sub> by means of reverse-phase liquid chromatography on silanized silica gel.

15 7. A process according to claim 2 for preparing a compound selected from antibiotic A 40926 complex, antibiotic A 40926 factor A, factor B and factor B<sub>0</sub> which comprises:

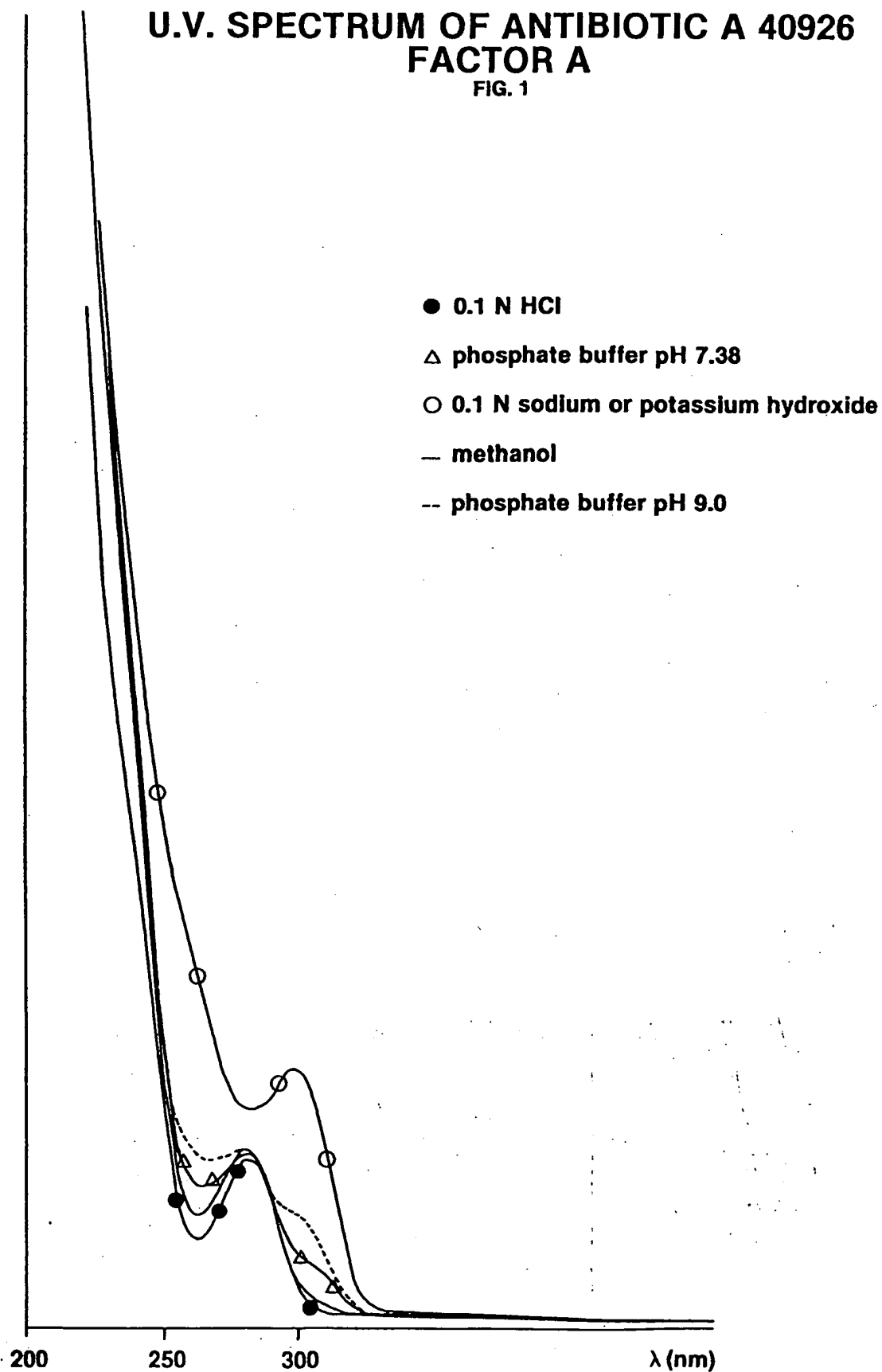
- 20 a) making the fermentation mass basic at a pH between 8.5 and 10.5
- b) filtering
- 25 c) acidifying the clear filtrate to pH 2.5-4.5
- d) filtering and discharging the filtrate
- e) suspending the filter cake in water and making it  
30 basic at a pH between 8 and 9
- f) after recovering the crude antibiotic A 40926 complex by filtration, subjecting it to affinity chromatography on immobilized D-Alanyl-D-Alanine

- g) optionally isolating antibiotic A 40926 factor A and factor B by means of partition, reverse-phase or ion-exchange chromatography, and
- 5 h) subjecting antibiotic A 40926 factor B to a further affinity chromatography procedure when antibiotic A 40926 factor B<sub>0</sub> is desired.
- 10 8. A process according to claim 7 wherein the chromatographic technique is reverse-phase chromatography and the stationary phase is selected from silanized silica gel and non-functionalized polystyrene resins.
- 15
9. A process according to claim 7 wherein the stationary phase is silanized silica gel pre-equilibrated with a buffered solution at a pH
- 20 between 4 and 9 and the eluent is a linear gradient mixture of a polar water-miscible solvent in the same buffered solution.
- 25 10. A compound of claim 1 for use as a medicine.
11. A pharmaceutical composition which comprises a compound of claim 1 in admixture with a pharmaceutically-acceptable vehicle.
- 30
12. The strain Actinomadura sp. ATCC 39727.

13. A biologically pure culture of the strain Actinomadura sp. ATCC 39727 or an antibiotic A 40926 producing mutant or variant thereof, capable of producing a compound of claim 1 when cultivated under submerged aerobic conditions in the presence of assimilable sources of carbon, nitrogen and inorganic salts.
- 5

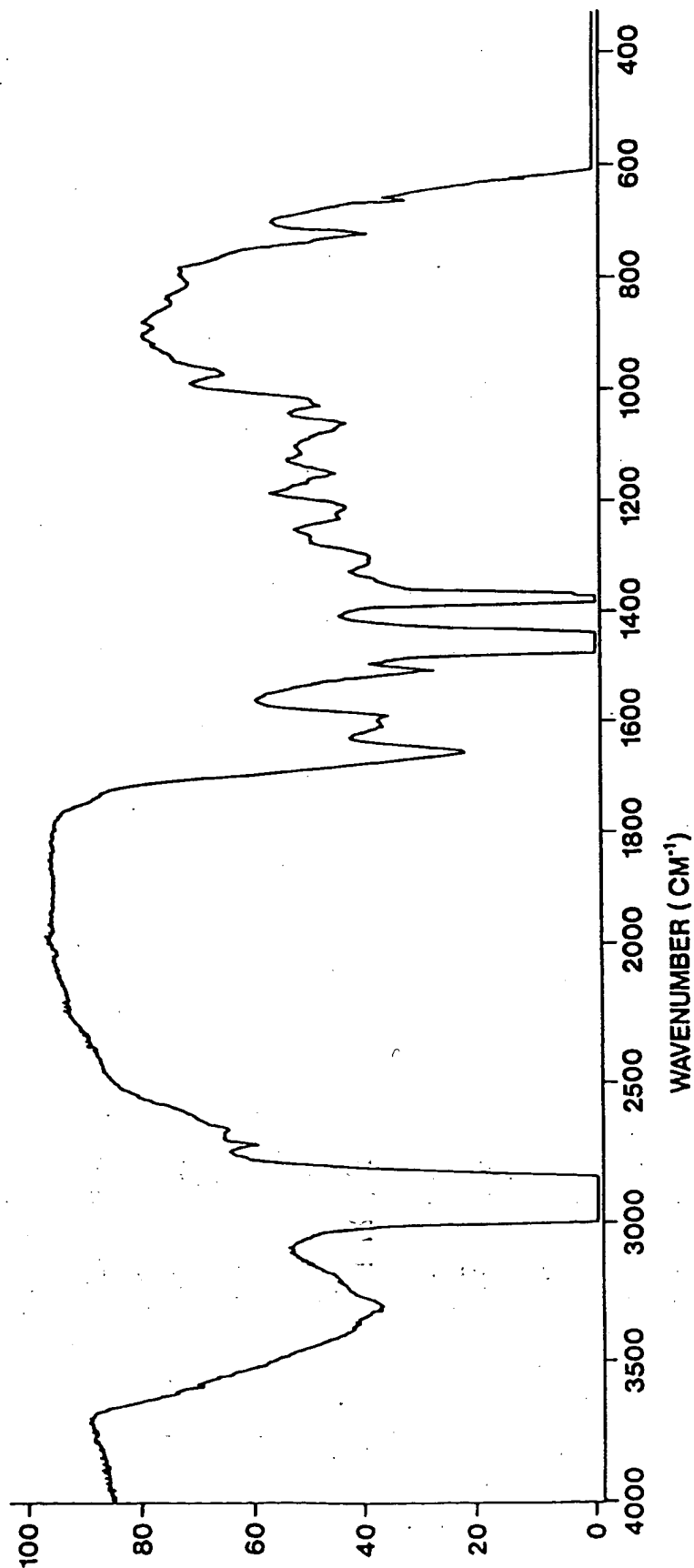
# U.V. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR A

FIG. 1



# I.R. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR A

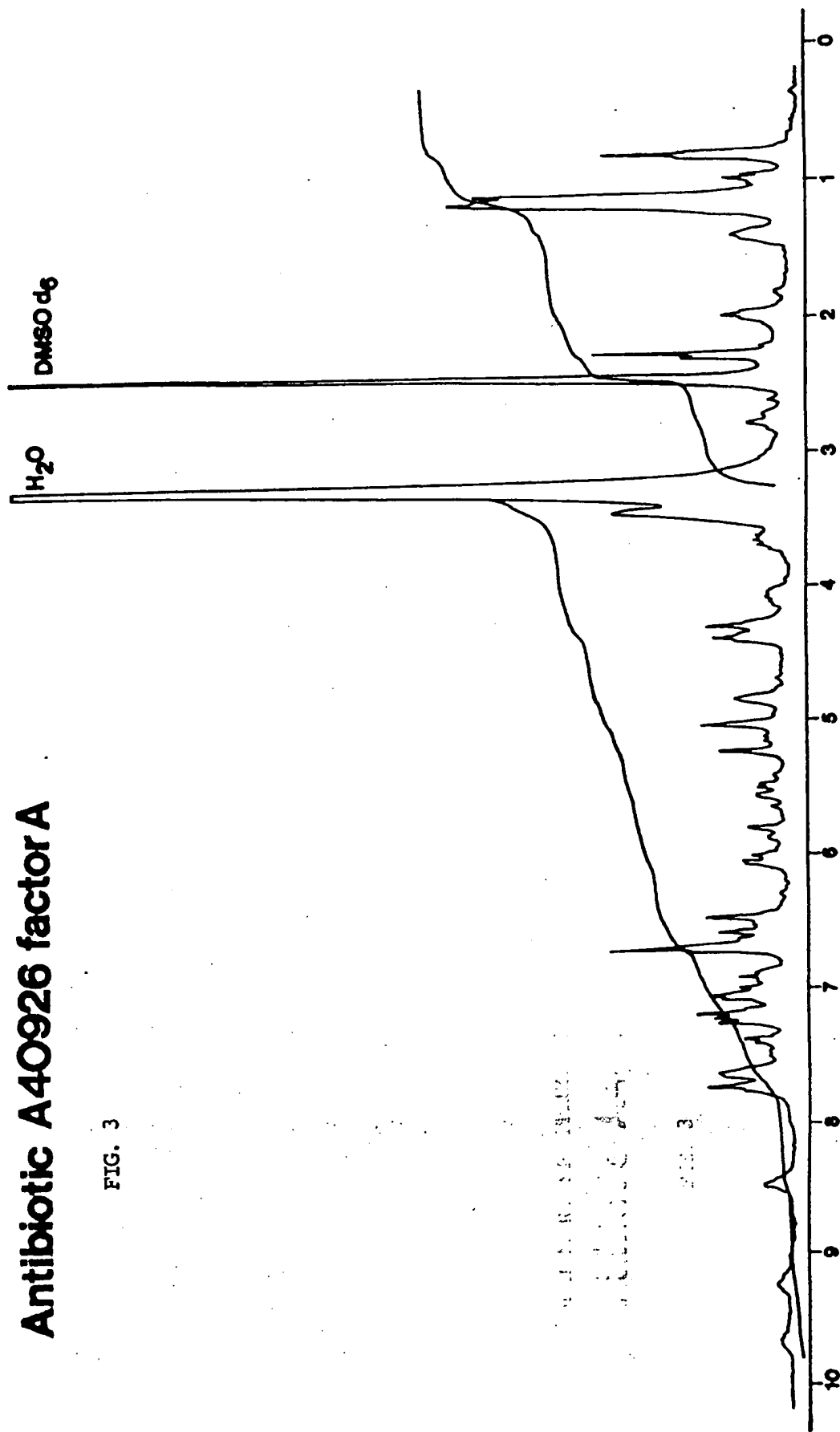
FIG. 2





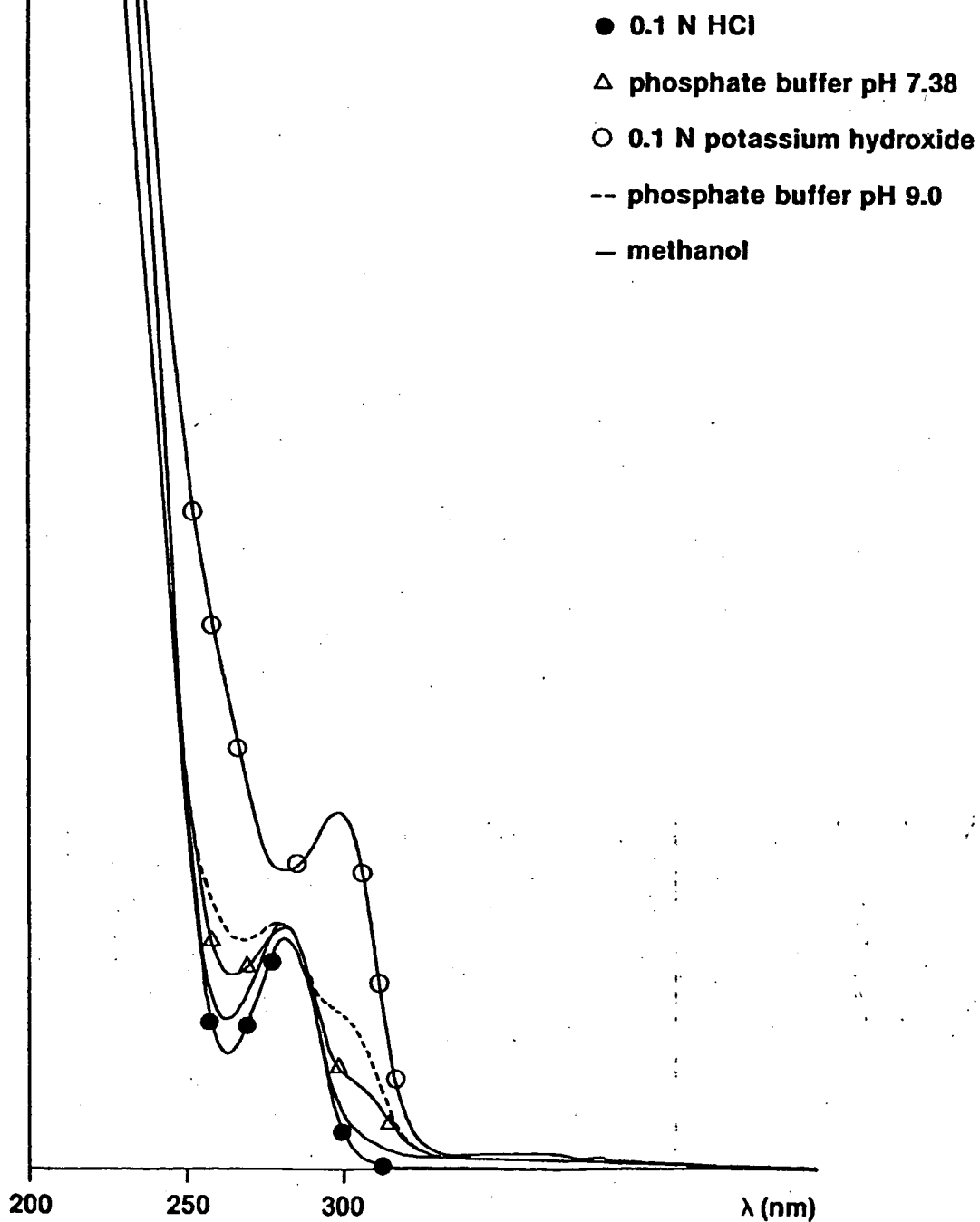
<sup>1</sup>H-N.M.R. SPECTRUM of  
**Antibiotic A40926 factor A**

FIG. 3



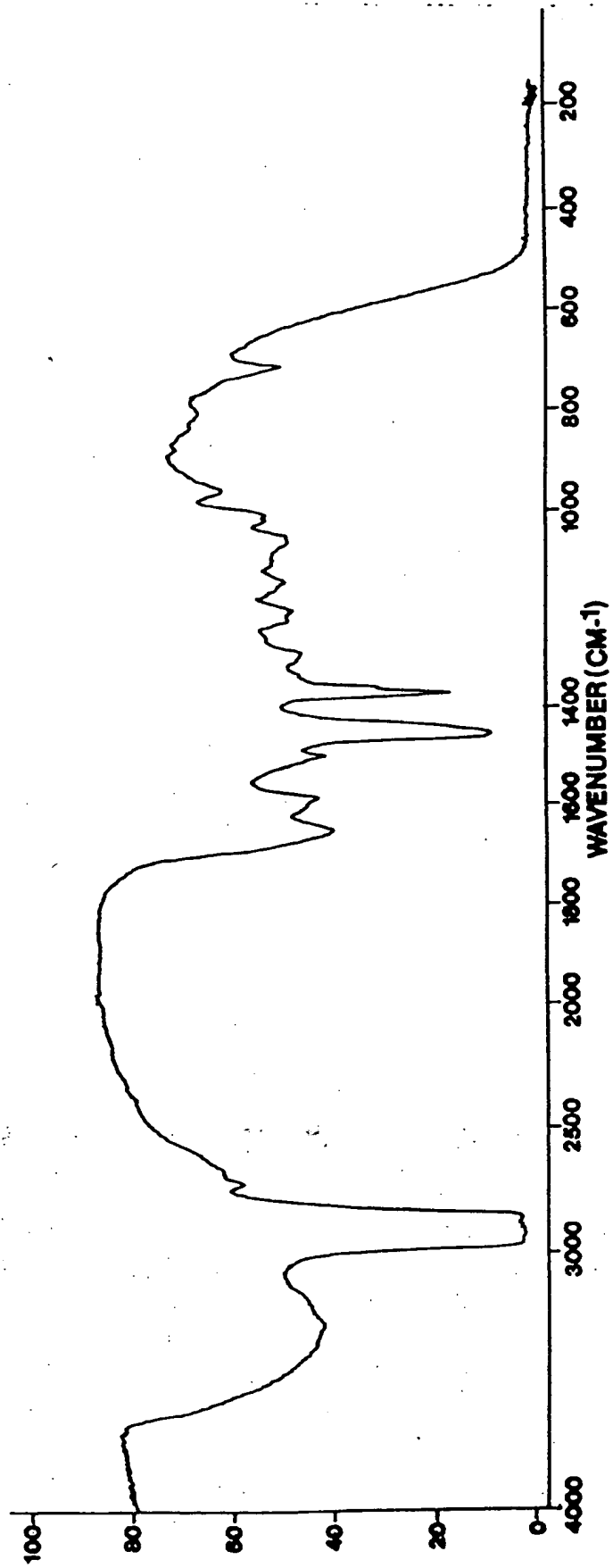
# U.V. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR B

FIG. 4



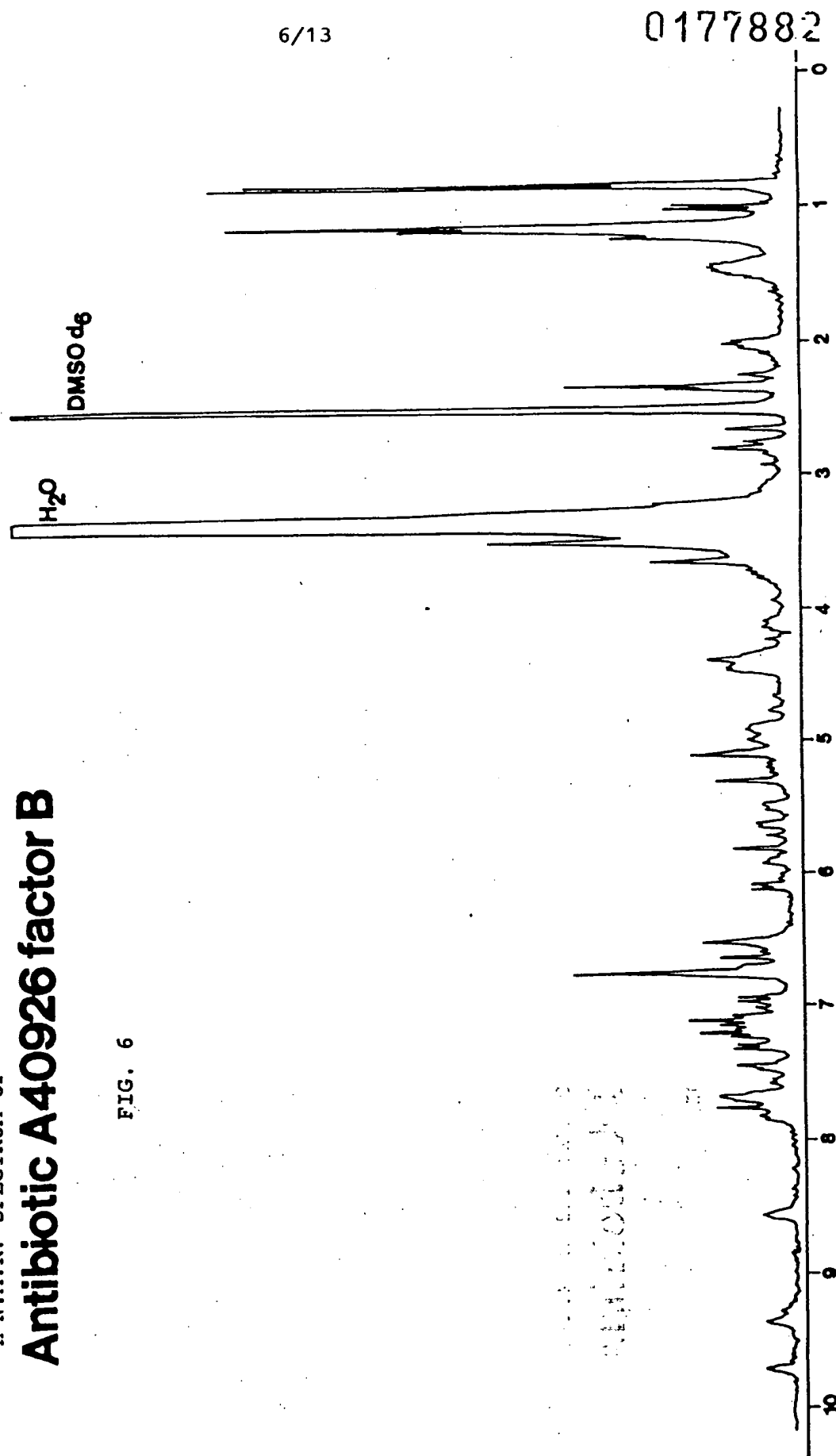
I.R. SPECTRUM OF  
**A40926 factor B**

FIG. 5



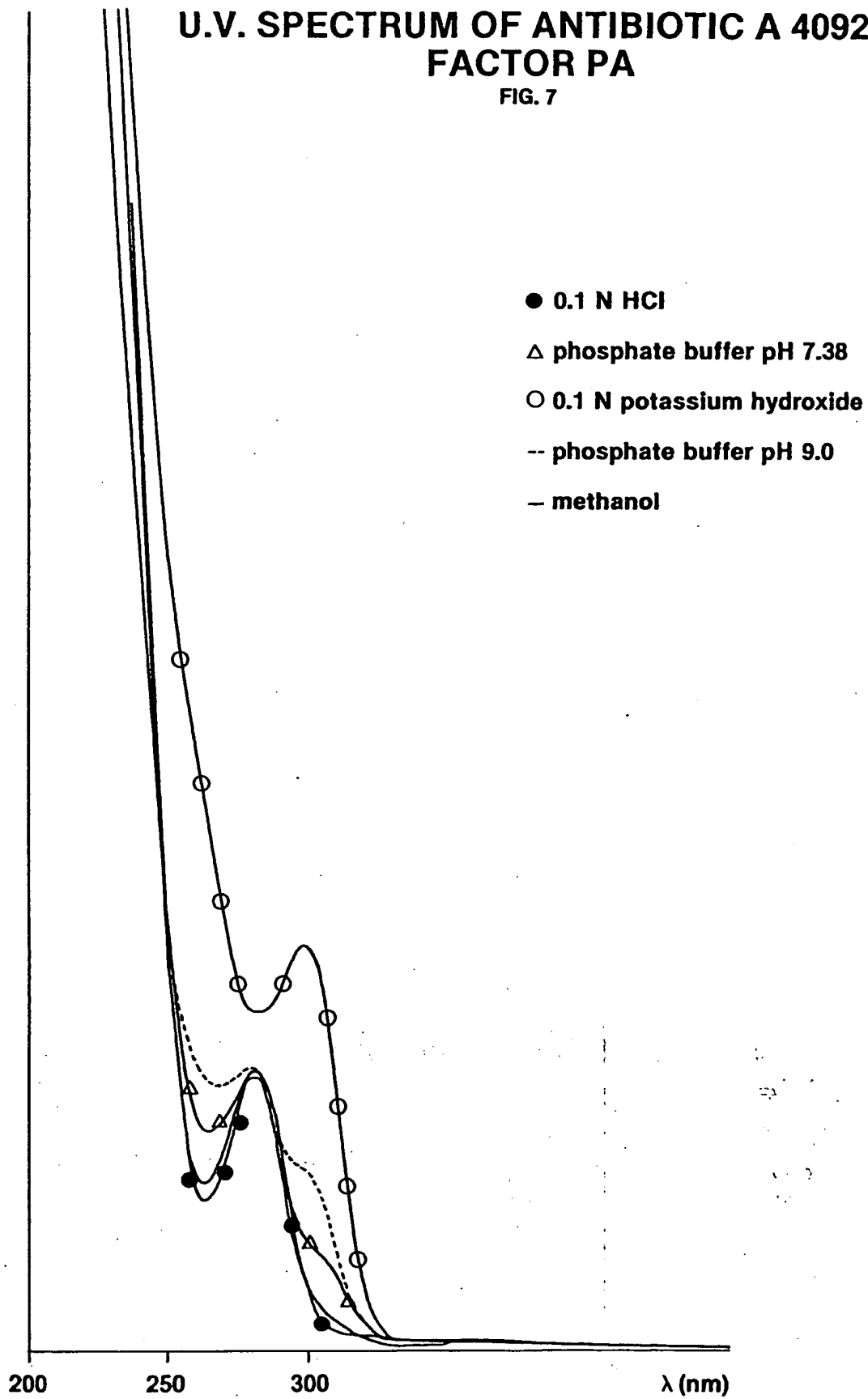
<sup>1</sup>H-N.M.R. SPECTRUM OF  
**Antibiotic A40926 factor B**

FIG. 6



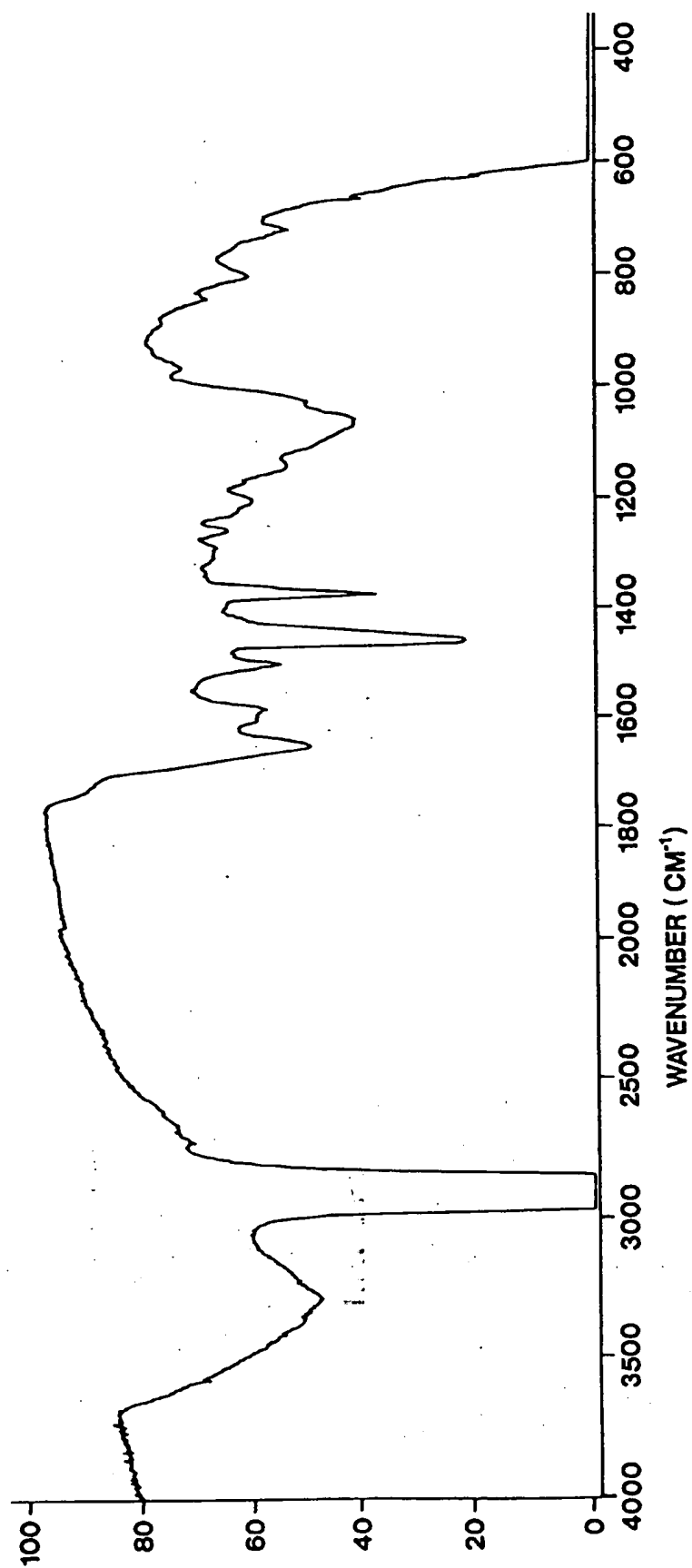
# U.V. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR PA

FIG. 7



I.R. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR PA

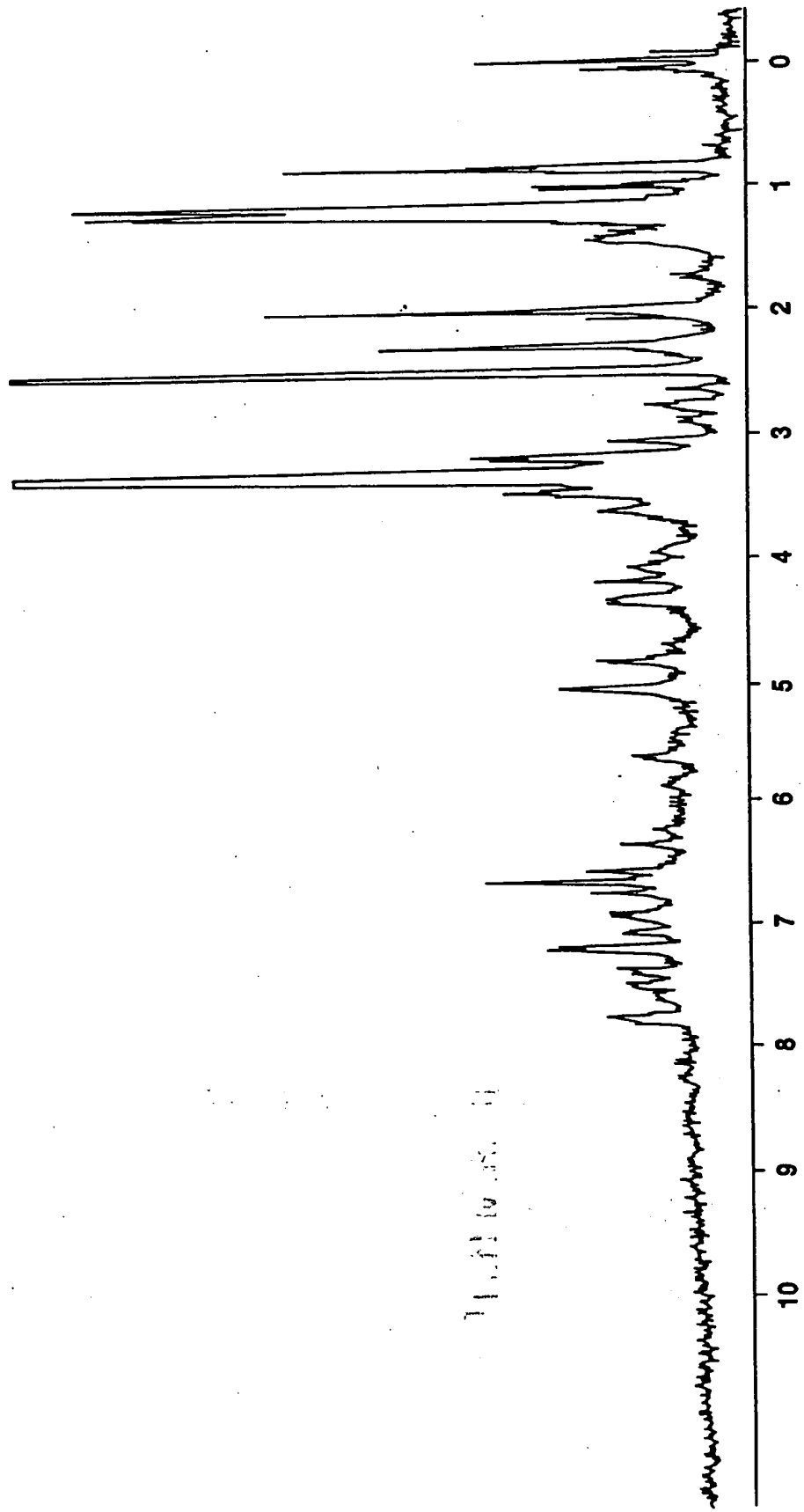
FIG. 8



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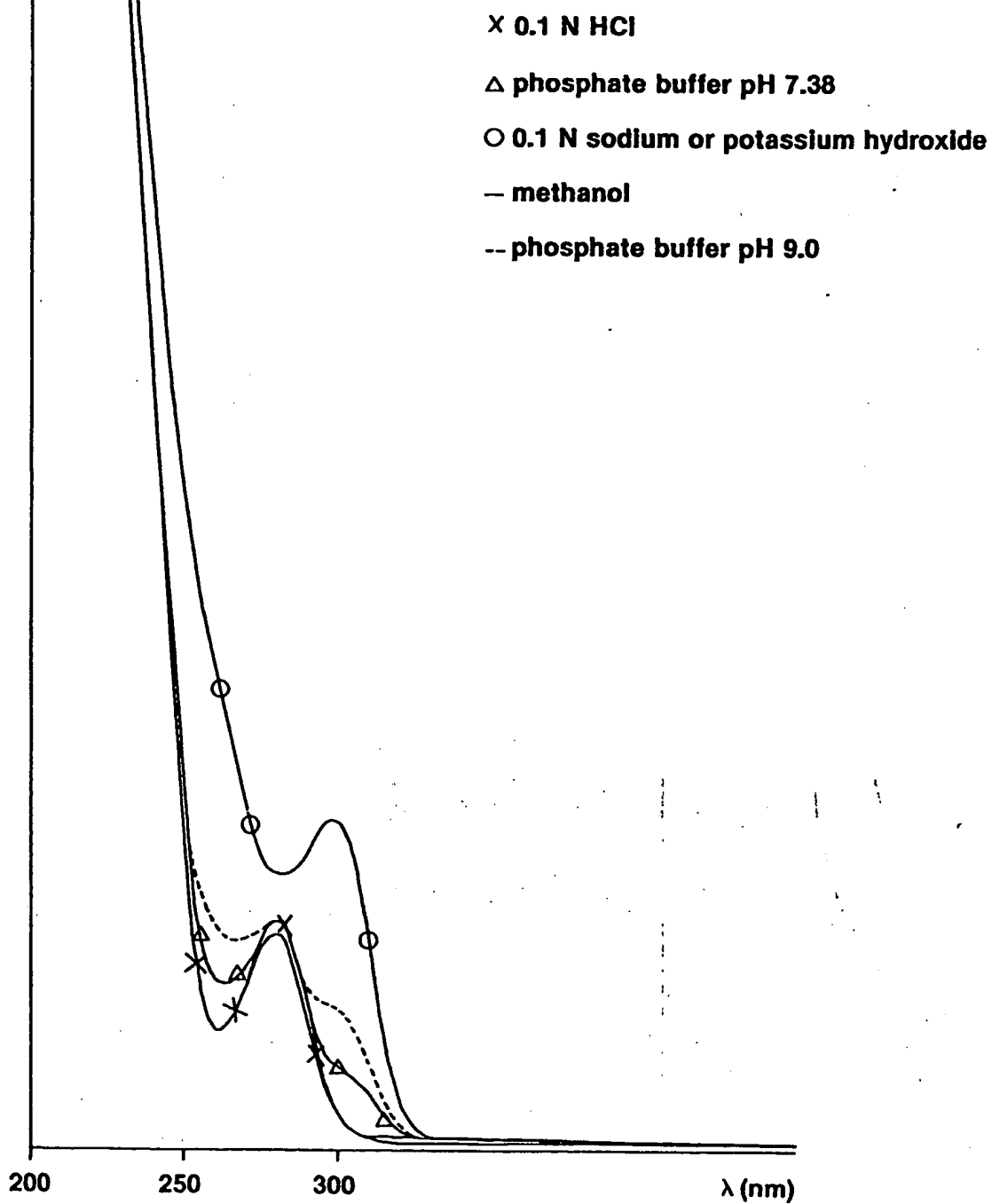
9/13

**$^1\text{H}$ .N.M.R. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR PA**  
FIG. 9



# U.V. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR PB

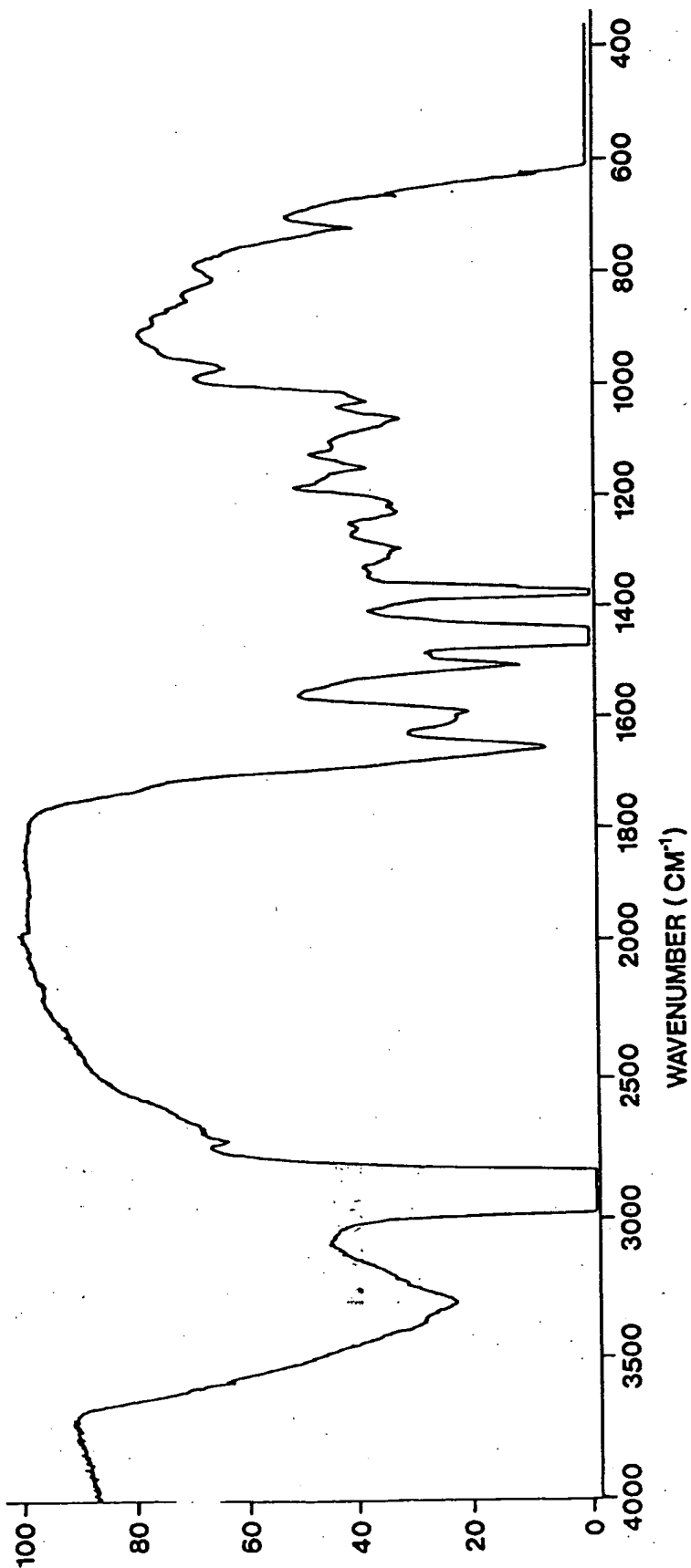
FIG. 10





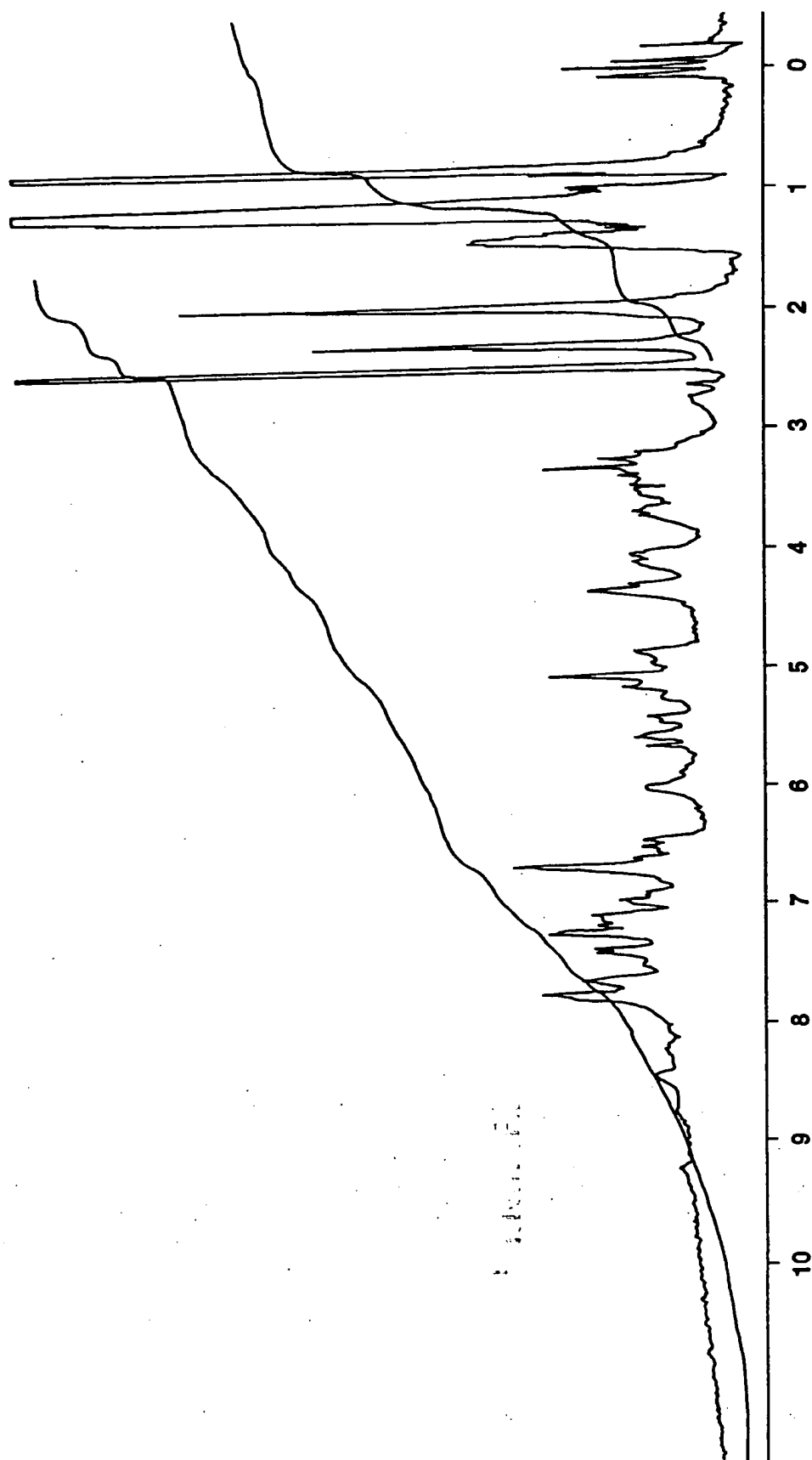
# I.R. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR PB

FIG. 11



**$^1\text{H}$ .N.M.R. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR PB**

**FIG. 12**



# <sup>1</sup>H.N.M.R. SPECTRUM OF ANTIBIOTIC A 40926 FACTOR PB

FIG. 13

